EXHIBIT 3

IN THE UNITED STATES DISTRICT COURT

EASTERN DISTRICT OF MICHIGAN

SOUTHERN DIVISION

TRUTEK CORP.,

Plaintiff,

v.

BlueWillow Biologics, Inc. ROBIN ROE 1 through 10, gender neutral fictitious names, and ABC CORPORATION 1 through 10 (fictitious names).

Defendants.

CIVIL ACTION No. 2:21-cv-10312-SJM-RSW

REPORT OF EDWARD A. LEMMO, Ph.D.

IN REPLY TO DEFENDANT'S EXPERT REPORT ON NON-INFRINGEMENT

My name is Edward Lemmo. I was engaged by counsel for Plaintiff, Trutek Corp. ("Trutek") as an expert witness in the above captioned matter where Trutek alleges that Defendant BlueWillow Biologics, Inc. ("BlueWillow") infringed on claims 1, 2, 6, and 7 of its U.S. Patent No. 8,163,802 ("the '802 Patent") by making and selling a product labeled Nanobio Protect.

On June 27, 2022, I submitted a document entitled, "Plaintiff's Opening Technical Report," in which I provided the following findings and conclusions:

1. BlueWillow's Nanobio Protect product is sold in the form of a liquid that is administered into a user's nasal passages.
2. The claims of Trutek's '802 Patent recite a method for nasally administering a formulation and for the formulation itself. The formulation of Trutek's '802 Patent is administered in and around a user's nasal passages.
3. BlueWillow's Nanobio Protect product exhibits an electrostatic charge, and once applied, creates an electrostatic field that extends from the skin or tissue of the user's nasal passages.
4. The formulation of Trutek's '802 Patent claims exhibits an electrostatic charge. Once applied, the formulation creates an electrostatic field in the vicinity of the user's nasal passages.
5. BlueWillow's Nanobio Protect product forms a thin film in the user's nasal passages. The thin film adheres to the skin or tissue of the user's nasal passages.

6. Trutek's '802 Patent claims that its formulation forms a thin film in and around the user's nasal passages. The thin film adheres to the skin or tissue of the user's nasal passages.

7. The formulation of BlueWillow's Nanobio Protect product contains at least one cationic agent. A cationic agent creates a positive electrostatic charge. According to BlueWillow's published literature and the Nanobio Protect product packaging, the formulation contains benzalkonium chloride. Benzalkonium chloride is a known cationic agent.

8. The formulation of BlueWillow's Nanobio Protect product contains at least one biocidic agent or biocide. A biocide is a substance that destroys or inhibits the growth or activity of living organisms. According to BlueWillow's published literature and the Nanobio Protect product packaging, the formulation contains benzalkonium chloride. Benzalkonium chloride is a known biocidic agent.

9. The Nanobio Protect product comprises nano-droplets that electrostatically attract and hold "germs" (*i.e.,* harmful particles). The nano-droplets further contain benzalkonium chloride (a known biocide) which renders the "germs" harmless.

10. Claims 1 and 2 of the '802 Patent recite a formulation that electrostatically attracts and holds harmful particles and renders them harmless.

11. The Nanobio Protect product reads upon the '802 Patent's method claim 1 when it is administered nasally because its formulation forms a thin film that it (1) adheres to the skin or tissue of the user's nasal passages; (2) electrostatically

attracts harmful particulate matter; (3) holds the harmful particulate matter; and (4) inactivates the harmful particulate matter and renders it harmless.

12. The Nanobio Protect product reads upon claim 2 of the '802 Patent's formulation because it (1) is intended to be applied to the skin or tissue of the user's nasal passages; (2) forms a thin film that adheres to the skin or tissue of the user's nasal passages; (3) comprises at least one cationic agent; (4) comprises at least one biocidic agent; (5) electrostatically attracts harmful particulate matter; (6) holds the harmful particulate matter; and (7) inactivates the harmful particulate matter, and renders it harmless.

13. The Nanobio Protect product reads upon claim 6 of the '802 Patent because it contains benzalkonium chloride, which is a cationic agent.

14. The Nanobio Protect product reads upon claim 7 of the '802 Patent because it contains benzalkonium chloride, which is a biocidic agent.

The remainder of my report provides the bases for my opinions expressed therein. Other than my restatement of my findings and conclusions (above), I shall not restate my opinions.

On August 15, 2022, defense expert, Mansoor M. Amiji, Ph.D. submitted a document entitled, "Responsive Expert Report of Mansoor M. Amiji, Ph.D. on Non-Infringement." In that document, Dr. Amiji criticized my findings and conclusions, and asserted that the Nanobio Protect product does not infringe the claims of the '802 Patent.

The purpose of my present submission is to rebut statements made by Dr. Amiji in his responsive expert report. Among the various comments made by Dr. Amiji regarding non-infringement of the NanoBio Protect product on the '802 Patent, Dr. Amiji states, "I have also considered the information provided in the Lemmo report dated June 27, 2022, the Ermakov report dated January 11, 2021, and the Burns report dated January 18, 2021, in addition to the other information and references described and discussed herein."

The gist of Dr. Amiji's criticism focuses on the validity of the works by Ermakov and Burns conducted separately in 2021. One of the key features of the '802 Patent claim 1 is the electrostatic attraction of particles from the air. This is a key feature of the patent that is expressed in claim 1, since it is the principal mechanism or method by which the technology can be applied to a product such as the Trutek/NasalGuard product.

The purpose of the 2021 Ermakov study was to determine the magnitude (amount) of the surface electrostatic charge created by means of application of solution, serum, and spray containing permanently ionized molecules. For each of the three nasal test products, a plain sheet of identical printer paper was used as a substrate. Each of these samples was prepared by coating the test product on a 1-inch square substrate by a product under test. Three samples for each item/product were tested identically, an average calculated and recorded. The surface charge was calculated using the following formula: Q=V*C/A, where Q is charge per unit area, V is measured voltage on the sensing electrode, C is capacitance, and A is the area of the sample under the sensing electrode.

The validity of the Ermakov test protocol was questioned by Dr. Amiji. In the study conducted by Dr. Ermakov, a Coulombmeter was used as the tool for measuring the electrostatic charge of the material. A Coulombmeter was used in combination with a Faraday cup (or a metal probe) for making charge measurements of a material. A Nanocoulombmeter is a Coulombmeter that is capable of measuring electrostatic charge down to the accuracy of a fraction of a nanocoulomb. This is a standard method of conducting this type of experiment. Electrostatic charge on an object can be measured by placing it inside a Faraday Cup. The charge is transferred to the cup and displayed on the meter's display. The Faraday Cup of the Coulombmeter has an outer, grounded metal shield that surrounds an inner electrode. The inner electrode, which is electrically isolated from the shield, is connected to a meter to measure the charge. The coulombmeter allows this electrostatic buildup to be easily measured simply by applying the instrument's probe to its lead. The Faraday cup is a metal (conductive) cup designed to catch charged particles in vacuum. The resulting current can be measured and used to determine the number of ions or electrons hitting the cup.

Dr. Ermakov findings indicate that 1) The test products i.e., Blank, NasalGuard Airborne Particle Blocker Gel, NasalGuard Misting Spray, and NanoBio Protect Solution, **all demonstrated the presence of a surface electrostatic charge**; and 2) **the surface electrostatic charge measured was determined to be approximately (in order of magnitude) similar in all three product samples tested.**

In 2021, Burns conducted a study to measure the surface electrostatic charge of nasal application products. The test equipment used in this trial was the ETS model 230 Nanocoulomb meter with a Faraday cup. The Model 230 measures electrical

charge directly, displaying the result in nanocoulombs. The evaluation of triboelectric charge generation is performed by measuring the amount of charge developed on a material. When used with a suitable Faraday cup, the charge on a wide range of material types and sizes can be measured accurately by the Model 230 Nanocoulombmeter. The Model 230 meets the requirements for charge measurement as specified in applicable ESDA, ASTM, EIA, DOD, as well as many other industry standards. The Model 230 is a complete instrument for measuring charge directly in nanocoulombs. This instrument may be used with a Faraday cup or pail, or a detector probe. It can also be used alone to measure the charge on capacitors or from capacitive discharge systems (with adequate protection).

The results of the Burns study demonstrated the presence of a surface electrostatic charge of similar order of magnitude between the Nasal Guard Spray product of Trutek and the Blue Willow NanoBio Protect Solution. **Both studies independently reached the same conclusions.**

Another comment made by Dr. Amiji in his report questioned the work of Ermakov and Burns measuring conductivity and not electrostatic charge. Dr. Amiji states, "[a]s an initial matter, both of the Ermakov and Burns tests measure the conductivity of the test formulations, not surface electrostatic charge."

The Encyclopedia of Separation Science is the most comprehensive resource available on the theory, techniques, and applications of separation science. The work presents information on three levels. The Encyclopedia of Separation Science is the first truly comprehensive work covering the whole of separation theory, methods, and techniques.

Salama, A.I.A., in the Encyclopedia of Separation Science, 2000 is the author of Mechanical Techniques: Particle Size Separation. Salama claims electrostatic charge represents an excess or deficiency of electrons on the particle surface. This charge may be assumed to reside on the particle surface in an absorbed gas or moisture film. The electrostatic charge generated on a particle is proportional to the particle surface area. This is the principle used in the design of electrostatic classifiers and precipitators. Furthermore, the presence of electrostatic charge on particle surfaces controls the behavior of particles in an electrostatic field.

CHARGE DENSITY MEASUREMENT THEORY

Electrostatic field strength is a direct measure of electrostatic charge. The electrostatic field strength of the samples was too small to measure directly. Instead, an ETS (Model 860) Wide Range Resistance Indicator was used to measure the resistivity of the card surfaces (both with and without the product coatings) in ohms per square, which is a unit for sheet resistance. It is a measure of the resistivity between parallel edges of a thin film resistive material. The film conductivity is the reciprocal of the resistivity. Electrical conductivity is the measure of a material's ability to conduct an electric current. When an electrical potential difference is placed across a conductor, the movable charges flow, giving rise to an electric current. The conductivity σ is defined as the ratio of the current density J to the electric field strength E. Therefore, the electrostatic field strength is expressed as: $J = \sigma E$.

Therefore, the electrostatic field strength is expressed as $E = J/\sigma$. Now, $E \propto 1/\sigma$. (i.e. the electrostatic field E is inversely proportional to the conductivity σ). Greater conductivity means lower electrostatic field strength. The charge density and

electrostatic field strength are directly proportional to one another. Therefore, the charge density and the electrical conductivity are inversely proportional to one another.

Conductivity of a nanoemulsion is usually measured to determine the stability of the nanoemulsion over time. In 2011, Bernardi *et.al.*, published a paper regarding the formation and stability of oil-in-water nanoemulsions containing rice bran oil: in vitro and in vivo assessments. A copy of the paper is attached to this report as Exhibit 1.

Dr. Amiji questioned the validity of using in vitro skin models. In his report, Dr. Amiji stated, "[i]n addition, neither of the Ermakov or Burns tests were conducted under circumstances that would mimic real life use of NanoBio Protect® in individuals. Both experiments were performed at room temperature, which is significantly lower than body temperature. Similarly, testing the surface charge of NanoBio Protect® as applied on paper and/or a piece of dried pig skin is not indicative or predictive of how the product will operate on human nasal skin or tissue. In this regard, I disagree with Dr. Lemmo's assertion that the "use of pig skin is more predictive than paper regarding how the product would behave on human skin," particularly in view of how Mr. Burns conducted the experiment."

In a review paper by Abd *et al.*, the review discusses the alternative skin models that have been developed as surrogates for normal and diseased skin and examines the concepts of using model systems for in vitro–in vivo correlation and the demonstration of bioequivalence. A copy of the paper is attached to this report as Exhibit 2.

UNDERSTANDING OF A PERSON OF ORDINARY SKILL

Dr. Amiji and I disagree on the level of education and experience of a person of ordinary skill. I will not re-argue my opinion in this matter. However in my forty-six years experience supervising pharmaceutical formulations, I have never encountered one with that level of education combined with experience. Typically, many of them have Bachelor's degrees or certifications. People with Dr. Amiji's qualifications would be performing developmental science and would have higher executive positions within a corporation.

Dr. Amiji expresses a number of opinions regarding the role of a person of ordinary skill in the matter of infringement. For example, in Paragraph 32, Dr. Amiji opines:

> *[I]n my opinion, a person of ordinary skill in the art reviewing the Ermakov and Burns testing would not understand the test results to establish that NanoBio Protect® satisfies the claim limitations and/or infringes the asserted claims of the '802 Patent.*

And, in Paragraph 52, he states:

> *Even if a person of skill in the art were to accept the results of the Ermakov and Burns testing as demonstrating that NanoBio Protect® exhibits an electrostatic charge when "applied to the skin or tissue of nasal passages" of an individual (a point which I dispute for the reasons explained above), the Ermakov and Burns testing does not demonstrate that the purported electrostatic charge operates to "inhibit[] harmful particulate matter from infecting an individual" or "electrostatically attracts the particulate matter to the thin film."*

As I understand it, the comprehension of test results or even of the claim language of the '802 Patent by a person of ordinary skill is not relevant to the issue of infringement. An activity of the Defendant or a product produced by the Defendant either infringes or it doesn't. That is an objective determination – not a subjective one.

ADMISSIONS MADE DIRECTLY BY BLUEWILLOW

The tests performed first by Ashok Wahi and then independently by Ermakov and Burns provided evidence to bolster Trutek's position that BlueWillow, via its Nanobio Protect product infringes the claims of the '802 Patent. In his critique of the opinions of Ermakov, Burns, and myself, Dr. Amiji's comments seek to deny, *inter alia*, that the Nanobio Protect product exhibits an electrostatic charge when applied to the skin or tissue of the nasal passages, or that there is an electrostatic attraction of the product's nano-particles to germs (*i.e.,* harmful particulate matter), or that the germs are inactivated by the benzalkonium chloride biocide contained in the nanoemulsion droplets. However, in doing so, he ignores the statements of admission made by BlueWillow itself on its website and its product packaging.

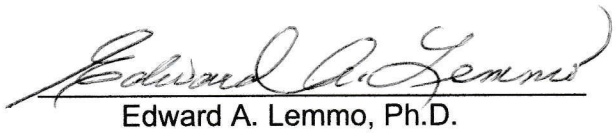
Dr. Amiji makes comments regarding the statements made by BlueWillow regarding their NanoBio Protect product. In my opinion, the admissions made by BlueWillow confirm infringement of the claims of the '802 Patent independent of the confirming tests. Samples of the BlueWillow website are attached as exhibits to my opening report. This following link provides admissions made by Blue Willow on the product: https://www.prnewswire.com/news-releases/bluewillow-biologics-launches-nanobio-protect-nasal-antiseptic-solution-301148517.html. A copy of the webpage is attached as Exhibit 3. A warning letter sent to BlueWillow by the Food and Drug Administration ("FDA") attests to the claims made by BlueWillow. A copy of this FDA generated letter is attached as Exhibit 4.

Nanobio Protect is produced as a nanoemulsion with nano-droplets that are positively charged. The nanoemulsion contains benzalkonium chloride, which is both a cationic agent and a biocidic agent. Exhibit D of my opening report states:

- *The Nanodroplets optimize the ability of the antiseptic to kill germs.*
- *The droplets sit on skin after application, enabling protection for up to 8 hours (in lab testing).*
- *Dry skin allows germs to penetrate. Nanodroplets hydrate the skin, preventing dryness and cracking.*
- *When bound to the oil-based Nanodroplets, the antiseptic does not irritate the skin.*

It is well-known that most harmful particles (including microorganisms) are negatively charged. It is basic physics that there would electrostatic attraction between the nano-droplets and the harmful particles. Once applied to the skin or tissue of the individual's nasal passages, the nano-droplets remain active in the nose for up to 8 hours. The Nanobio Protect product adheres to the nasal tissue in a tin film. If this were not so, the liquid would immediately drip out. The nanoemulsion becomes impermeable, not allowing "germs to penetrate." The "germs" become in contact with the benzalkonium chloride biocide and are held there long enough to be deactivated and rendered harmless. In my opinion, considering the written admissions of the properties of the Nanobio Protect product as well as the tests performed in the laboratory to confirm those claims, the Nanobio Protect product reads on claims 1, 2, 6, and 7 of the '802 Patent.

Dated: September 29, 2022



Edward A. Lemmo, Ph.D.

EXHIBIT 1

Bernardi *et al. Journal of Nanobiotechnology* 2011, **9**:44
http://www.jnanobiotechnology.com/content/9/1/44

JOURNAL OF NANOBIOTECHNOLOGY

RESEARCH Open Access

Formation and stability of oil-in-water nanoemulsions containing rice bran oil: *in vitro* and *in vivo* assessments

Daniela S Bernardi[1*], Tatiana A Pereira[1], Naira R Maciel[1], Josiane Bortoloto[1,2], Gisely S Viera[1], Gustavo C Oliveira[1] and Pedro A Rocha-Filho[1]

Abstract

Background: Nanoemulsions have practical application in a multitude of commercial areas, such as the chemical, pharmaceutical and cosmetic industries. Cosmetic industries use rice bran oil in sunscreen formulations, anti ageing products and in treatments for skin diseases. The aim of this study was to create rice bran oil nanoemulsions using low energy emulsification methods and to evaluate their physical stability, irritation potential and moisturising activity on volunteers with normal and diseased skin types.

Results: The nanoemulsion developed by this phase diagram method was composed of 10% rice bran oil, 10% surfactants sorbitan oleate/PEG-30 castor oil, 0.05% antioxidant and 0.50% preservatives formulated in distilled water. The nanoemulsion was stable over the time course of this study. *In vitro* assays showed that this formulation has a low irritation potential, and when applied to human skin during *in vivo* studies, the nanoemulsion improved the skin's moisture and maintained normal skin pH values.

Conclusion: The results of irritation potential studies and *in vivo* assessments indicate that this nanoemulsion has potential to be a useful tool to treat skin diseases, such as atopic dermatitis and psoriasis.

Background

Nanoemulsions are obtained when the size of an emulsion globule reaches approximately 20-500 nm. The small droplet size can resist the physical destabilisation caused by gravitational separation, flocculation and/or coalescence. It also avoids the creaming process because the droplet's Brownian motion is enough to overcome the gravitational separation force [1,2]. The size and polydispersity of nanoemulsions can affect properties such as particle stability, rheology, appearance, colour, texture and shelf life [3]. In nanoemulsions, the most frequent instability phenomenon is Ostwald ripening [4,5], which can be calculated according to the Lifshitz-Slezov and Wagner theory (LSW) using the following equation [6,7]:

$$\omega = \frac{dr^3 n}{dt} = k\frac{Dc_\infty \gamma M}{\rho^2 RT} \quad (1)$$

Where ω is defined as the rate of change of the cube of the number average radius, D is the diffusion coefficient of the dispersed oil phase in the aqueous phase, γ is the interfacial tension between the two phases, c_∞ is the bulk solubility of the oil in the water and ρ is the oil density. k is a constant that has the value of 8/9 in the LSW.

Nanoemulsions are well characterised and are a promising drug delivery system with practical applications for pharmaceutical, cosmetic and chemical industry applications. They have been used in intravenous, oral and ocular drug administrations and have reduced drug side effects and improved the pharmacological effects of the drugs given [8-10,4]. Nanoemulsions are primarily produced either by high-energy emulsification (e.g., high-pressure homogenisation) or by low-energy emulsification (using physicochemical properties of the components) [11]. This work focuses on the latter method for nanoemulsion synthesis.

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Rice (*Oryza sativa*) bran oil has unsaponifiable fractions that contain high levels of antioxidant-rich components, such as tocopherols/tocotrienols and gamma-oryzanol, which could be useful for topical formulations [12]. The cosmetics industry has used rice bran oil in sunscreen formulations [13], in topical aging prevention products [14] and in treatments for skin diseases [15].

When skin is affected by diseases such as atopic dermatitis and psoriasis, it exhibits a compromised skin barrier function that causes increased transepidermal water loss [16-18]. Moisturisers can help improve the skin's function by relieving the cutaneous manifestations of these diseases [18-20]. Measuring the *stratum corneum* (SC) hydration degree gives important information about the biophysical properties and the function of the skin [21].

In vitro studies, such as the HET-CAM (Hen's Egg Test on the Chorioallantoic Membrane), are used to evaluate cosmetics products by immediately showing whether or not a solid or liquid substance irritates the hen's egg chorioallantoic membrane [22].

The aim of this study was to obtain nanoemulsions of rice bran oil and to evaluate their physical stability, irritating potential and *in vivo* moisturising activity.

Materials and methods

Materials

Sorbitan oleate (HLB 4.3) and rice bran oil were kindly provided from Lipo do Brasil (Brazil). PEG-30 castor oil (HLB 11.7) was donated by Oxiteno (Brazil). The preservative DMDM Hydantoin (and) Iodopropynyl Butylcarbamate (Glydant® plus) was obtained from Chemyunion (Brazil), and the antioxidant Butyl Hydroxy Toluene (BHT) was purchased from Synth (Brazil).

Preparation of nanoemulsion

The following surfactant mixtures were evaluated to find a stable nanoemulsion formulation: polysorbate 80/sorbitan oleate, polysorbate 60/sorbitan oleate, ceteareth-20 OE/ceteth-2, PEG-15 castor oil/sorbitan oleate, PEG-30 castor oil/sorbitan oleate and PEG-40 castor oil/sorbitan oleate. The PEG-30 castor oil/sorbitan oleate ($HLB_{resulting}$= 8.0) was the only surfactant combination that resulted in a stable formulation and therefore was chosen for further study.

The phase diagram method was used with different concentration of constituents to result in 42 different emulsion formulations. All emulsions were prepared according the Emulsion Phase Inversion (EPI) method, where the water and oil phases were heated separately at 75°C, the water phase was added into the oil phase (rice bran oil and surfactants) while stirring at 600 rpm (Fisatom, 713-Dmodel, Brazil), and the mixture was then cooled to 25°C while stirring.

Determination of nanoemulsion droplet size

The mean droplet size and polydispersity index of the nanoemulsions were determined by dynamic light scattering (DLS) (Zetasizer, modelo ZS, Malvern Instruments, UK). Measurements were performed at 25°C using a scattering angle of 90°. Samples were considered polydisperse when the polydispersity index was higher than 0.2 [23].

Stability study

The preliminary stability of the nanoemulsion preparation was evaluated at 24 hours by centrifuge and thermal stress analyses. Stability was assessed by macroscopic emulsion observation and droplet size analysis. The purpose of these tests was to select a stable, low-surfactant formulation with a nanoemulsion-size droplet and stable physicochemical properties. The selected nanoemulsion was prepared in triplicate, and the samples were stored at 25 ± 2°C, 40 ± 2°C and 5 ± 2°C. Tests were performed at 24 hours, 7, 15, 30, 60 and 90 days after preparation. The analysis measurements were droplet size, pH value and electrical conductivity.

Physical-chemical analyses

To perform the thermal stress test, nanoemulsions were heated in an ultra bath thermostat (Nova Técnica, Brazil) at temperatures ranging from 40 to 80°C. The temperature was increased by 5°C every 30 minutes. The nanoemulsions were centrifuged (Fanem 206-R, Brazil) at 1000, 2500 and 3500 rpm (70, 440 and 863 G, respectively) for 15 minutes in each rotation to accelerate possible instability phenomena. A pH meter (PM608 model - Analion, Brazil) was used to measure the pH of the nanoemulsions at 25 ± 2°C. The electrical conductivity was evaluated at 25 ± 2°C by a portable conductivity meter (mCA-150 model, Tecnopon, Brazil).

Irritant test in an organotypic model - HET-CAM (Hen's Egg Test on the Chorioallantoic Membrane)

The HET-CAM test is routinely used to evaluate the potential eye irritation of raw materials but can in some cases be used to evaluate skin irritation, e.g. in the case of surfactants. Irritation causes alterations in the vascular system of the HET-CAM that result in membrane discoloration, haemorrhaging and increased perfusion. The method used in this manuscript is a modification of the method described by Luepke [24] and adapted by Mehling [25] that allows the immediate evaluation of irritation by solid or liquid substances in the hen's egg chorioallantoic membrane. Each substance was tested on three fertilised eggs that were incubated for 9 days prior to testing. The CAM (Chorioallantoic Membrane) was exposed to 300 μL of one of the following

substances: (1) nanoemulsion (pH 6.54), (2) surfactant solution blend (5% sorbitan oleate, 5% PEG-30 castor oil and 90% water (pH 6.32), (3) Sodium lauryl sulphate (SLS) 10% w/w (positive control, pH 6.05) and (4) saline solution (negative control, pH 6.0). The CAM was rinsed with physiological saline solution after 30 seconds of exposure to each substance, and the intensity of the reactions (hyperaemia, haemorrhage and coagulation) was semi-quantitatively assessed on a scale of 0.5, 2 and 5 minutes after treatment; longer observation times give no additional important information. The numerical time-dependent scores for hyperaemia, haemorrhage and coagulation are summed to give a single numerical value indicating the irritation potential of the test substance on a scale with a maximum value of 21. The mean value of four tests makes possible an assessment by a classification scheme analogous to the Draize categories (Table 1).

In vivo assessment

Approval to conducted the *in vivo* studies was obtained from the Ethics Committee of Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Ribeirão Preto, São Paulo, Brazil, under protocol number CEP/FCFRP n°. 147.

Seventeen Caucasian volunteers with healthy skin (all women, 20-29 years old), five patients with atopic dermatitis (all women, 21-30 years old), and four psoriasis patients (1 man and 3 women, 28-56 years old) without skin lesions on the forearm were included in the study. The forearm area was washed with mild soap two hours before the analysis. The volunteers were kept for 10 min in a controlled room at ambient temperature (25 ± 2°C; 29-34% relative humidity) before starting the assay. Baseline values were determined using a Corneometer CM 820, a Sebumeter SM 810 and a Skin-pH-meter PH 900 instruments (Courage &Khazaka, Köln, Germany) before applying the formulation. Then, 50 μL of nanoemulsion was applied to the right forearm in three rectangular areas of 13.80 cm^2 and distributed for 20 seconds by rubbing the test area using a circular motion. Excess nanoemulsion was left on the skin. The treated skin of each patient was measured 30, 60, 90, 120 and 150 minutes after nanoemulsion application, with one measure for sebum content and triplicate measurements of electrical capacitance and skin pH.

Table 1 Classification of cumulative scores in the chorioallantoic membrane test (According Luepke 1985 [24])

Cumulative Score	Irritation assessment
0-0.9	Practically none
1-4.9	Slight
5-8.9	Moderate
9-21	Strong

Results and discussion

Preparation of nanoemulsion

The phase diagram with the rice bran oil, surfactants sorbitan oleate/PEG-30 castor oil and water shows formation of five different areas: (I) O/W (Oil/Water) nanoemulsion, (II) phase separation, (III) gel phase, (IV) W/O (Water/Oil) emulsions and (V) O/W emulsions (Figure 1).

Table 2 shows the composition and the droplet size of the nanoemulsion systems.

All of the nanoemulsions (Table 2) were stable when tested using the centrifugation test. The only formulation that showed signs of instability at high temperatures (70°C) was the nanoemulsion composed of 10:20:70 (oil, surfactants and water, respectively).

A surfactant concentration of 5.00% was not sufficient to form a nanoemulsion, even with smaller amounts of oil. The surfactant amount affects the stabilisation and size of the emulsion droplets. From the experimental results, the nanoemulsion that used the lowest possible surfactant concentration while still maintaining thermal stability, centrifugal stability and small droplet size was selected as the working formulation.

Based on these preliminary results, we chose a formulation composed of 10% rice bran oil, 10% surfactant blend and 80% water and proceeded with further stability tests and *in vitro* and *in vivo* evaluations.

Stability study

The formulation composed of 10:10:80 (rice bran oil, surfactant blend and water) was supplemented with 0.05% antioxidants and 0.50% preservatives. The formulation was tested at three different storage temperatures: 25 ± 2°C, 40 ± 2°C and 5 ± 2°C. By granulometric analysis the particles maintained a mono-disperse, monomodal peak after 24 hours (Figure 2). The formulation was stable for up to 90 days as determined by macroscopic analysis.

Droplet size measurements are a good indicator of the formulation stability. A fast droplet size increase indicates low system stability. The droplet size for this formulation remained constant over 90 days for all temperature conditions (Figure 3).

The nanoemulsions had polydispersity index values below 0.2 throughout the 90-day testing period, indicating the high fidelity of the system (low polydispersity), which may reflect the overall stability of this formulation and synthesis method. Polydispersity values near 1.0 are indicative of a polydisperse system [26]. The long term stability of nanoemulsions was previously evaluated and was also verified by stability studies conducted over three months. The W/O nanoemulsion produced by low energy emulsification showed no difference in droplet size over the study period at both 25°C and 4°C [27]. The W/O nanoemulsion demonstrated high physical stability,

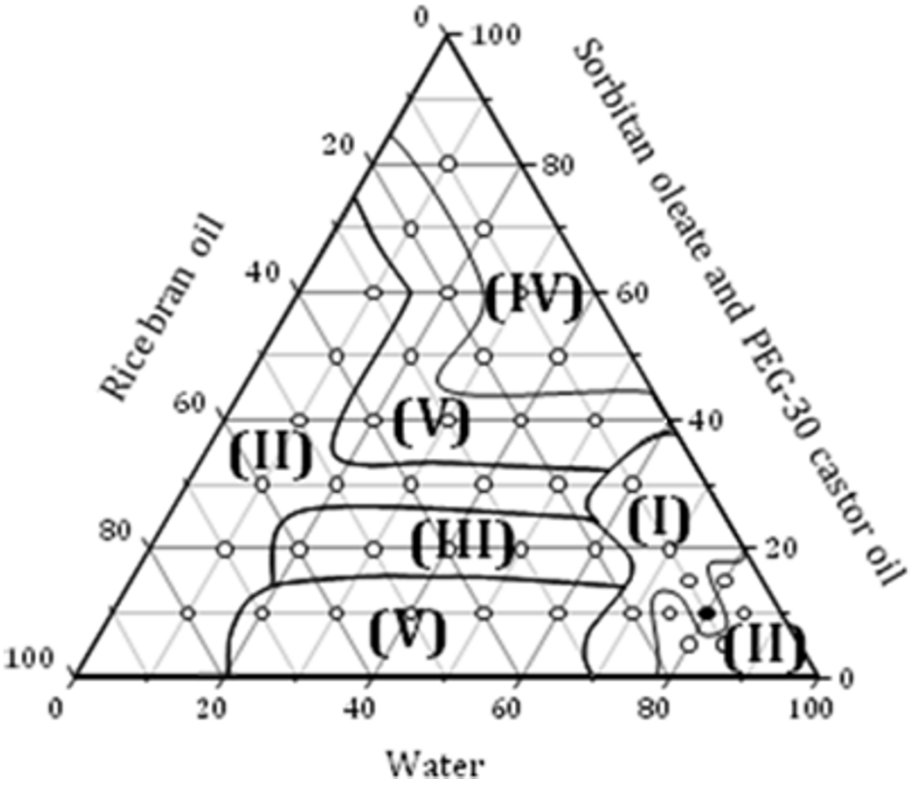

Figure 1 Phase diagram with rice bran oil, sorbitan oleate/PEG-30 castor oil and water. Region I: nanoemulsion, II: phase separation, III: gel phase, IV: W/O emulsions; V: O/W emulsions.

corroborating our results for temperatures of 5 ± 2°C and 25 ± 2°C.

Low-energy emulsification is better at producing stable nanoemulsions than its higher energy counterpart. When nanoemulsions were prepared using a high pressure homogeniser, the droplet size was initially around 100 nm; however, the particles increased in size after 30 days at either 25 or 4°C. This phenomenon was attributed to the preparation method [28]. The low-energy emulsification method used in our study showed high stability with respect to the droplet size and polydispersity index.

pH value determination

Monitoring the pH value is important for determining the emulsions' stability because pH changes indicate the occurrence of chemical reactions that can compromise the quality of the final product. Emulsions produced with vegetable oils may experience a decrease in pH due to the hydrolysis of fatty acid esters into free fatty acid degradation products [29].

The nanoemulsions had stable pH values for almost all conditions tested (Figure 4). Only at a temperature of 40 ± 2°C and 90 days of incubation was there a statistically significant decrease in the pH of the nanoemulsion. The high temperature might have destabilised the nanoemulsion by hydrolysis, but it did not affect the overall quality of the nanoemulsions because the pH values remained around pH 6.0, which is an acceptable, non-skin irritating pH value.

Electrical conductivity

The nanoemulsion showed changes in electrical conductivity at all storage conditions (Figure 5).

Table 2 Composition of formulation characterised as nanoemulsions

Rice bran oil (% w/w)	Sorbitan oleate/PEG-30 castor oil (% w/w)	Purified water (% w/w)	Droplet size (nm) ± (Standard Deviation)
10.00	30.00	60.00	91 ± 19
10.00	20.00	70.00	45 ± 12
10.00	**10.00**	**80.00**	**69 ± 17**
20.00	10.00	70.00	303 ± 32
10.00	15.00	75.00	121 ± 13

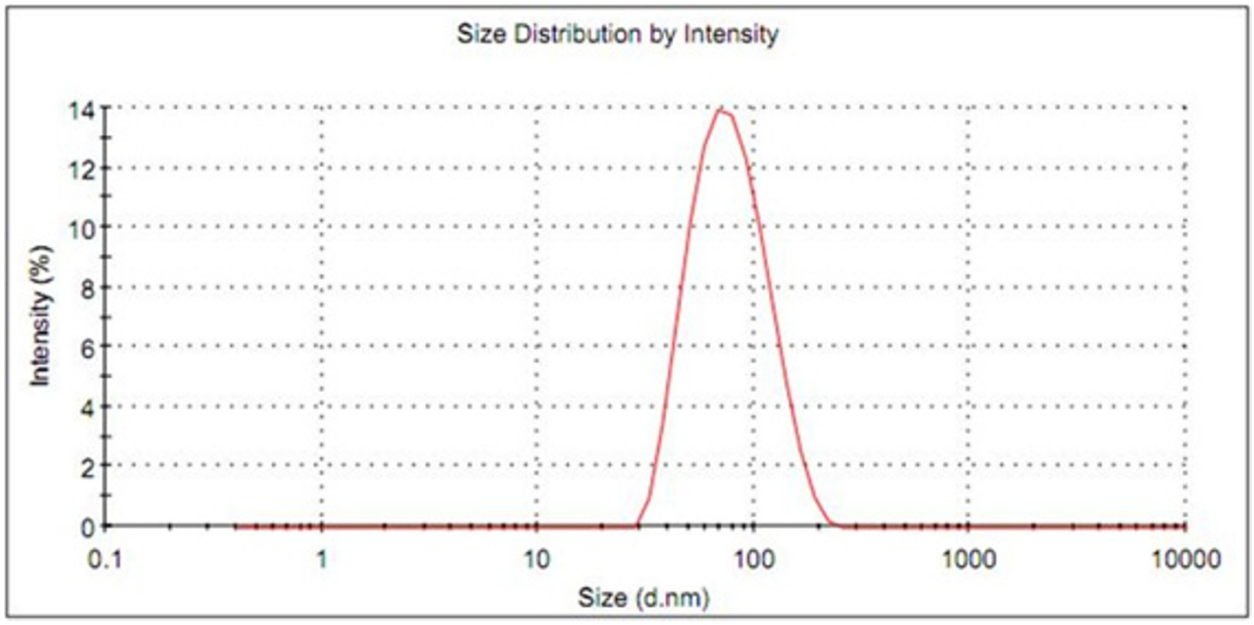

Figure 2 Droplet size distribution of nanoemulsions after 24 hours.

Changes in the electrical conductivity can indicate nanoemulsion instability and may influence the nanoemulsion droplet size [30]. In these studies, changes in electrical conductivity did not affect the nanoemulsion droplet size (Figure 3). It is difficult to assess the emulsion stability solely by electrical conductivity because the relationship between an increase in electrical conductivity and emulsion instability is not linear [31].

Thus, we could not conclusively determine the nanoemulsion's stability by this parameter. However, because the particle size and the pH value did not significantly change across different conditions, we considered our nanoemulsion to be stable. Nanoemulsion stability is a crucial parameter in determining the moisturising activity of the nanoemulsions *in vivo*.

Irritant test in an organotypic model - HET-CAM (Hen's Egg Test on the Chorioallantoic Membrane)

Topical application products must have a low ocular/mucous membrane and a low dermal irritation potential.

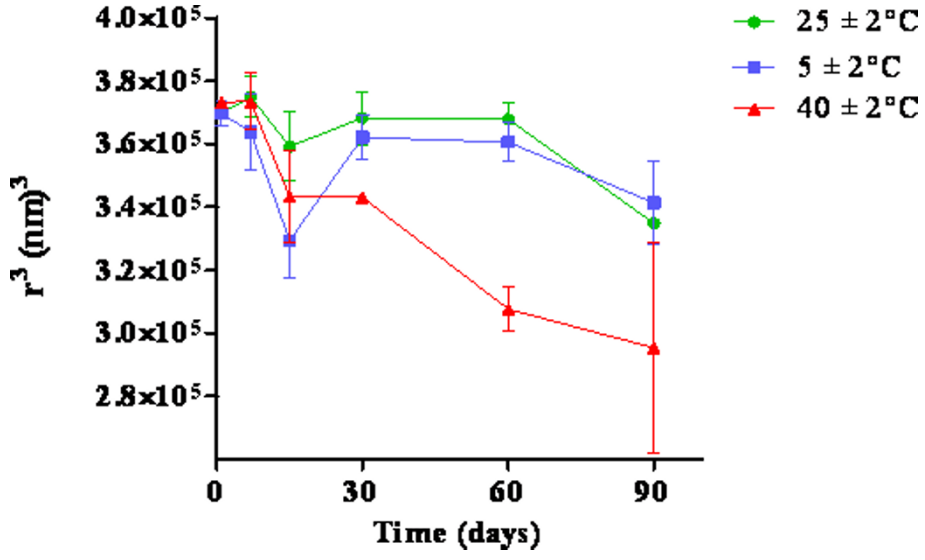

Figure 3 Nanoemulsion droplet size under different storage conditions during a 90-day stability test.

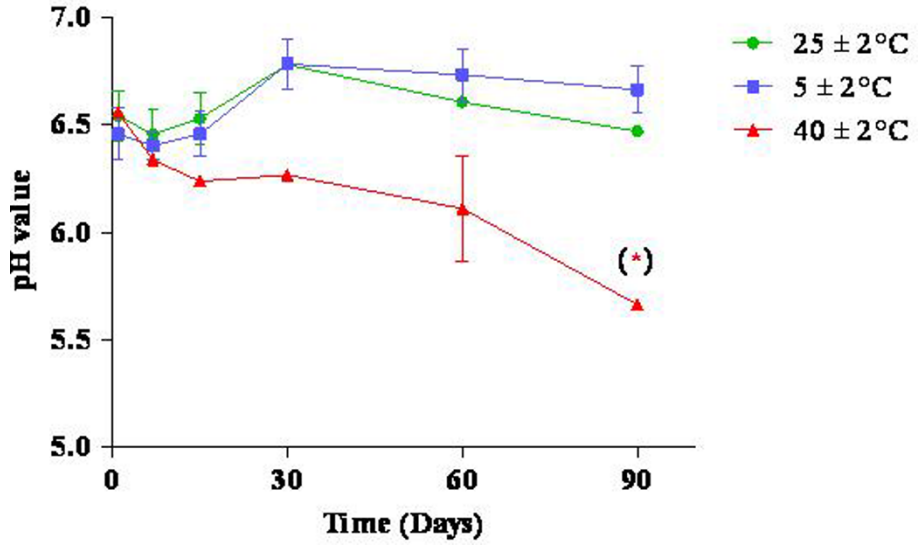

Figure 4 pH values for nanoemulsions over time under different storage conditions.

The irritation potential depends on the concentration of the substance as well as the chemical composition and the pH of the formulation [25].

The HET-CAM test can help evaluate the irritation potential of substances *in vitro* and *in vivo* [32]. The CAM showed no signs of irritation after application of either the nanoemulsion or the negative control substance, so the nanoemulsion was therefore considered practically non-irritating. The surfactant solution by itself caused mild hyperaemia, which suggests that the presence of rice bran oil in the nanoemulsion may have protected the chorioallantoic membrane from the irritating effects of the surfactant solution (Table 3). The pH values were the same for all samples tested to eliminate pH as a variable in the HET-CAM results.

Previous studies showed that O/W microemulsions containing linoleic acid were only barely irritating in the HET-CAM test, indicated by a slight discoloration of the chorioallantoic membrane [33]. The HET-CAM test showed that the nanoemulsion containing rice bran oil was essentially non-irritating.

In vivo assessment

The formulation composed of 10:10:80 (rice bran oil, surfactant blend and water) was chosen for the *in vivo* study due to its high stability and lack of irritation in

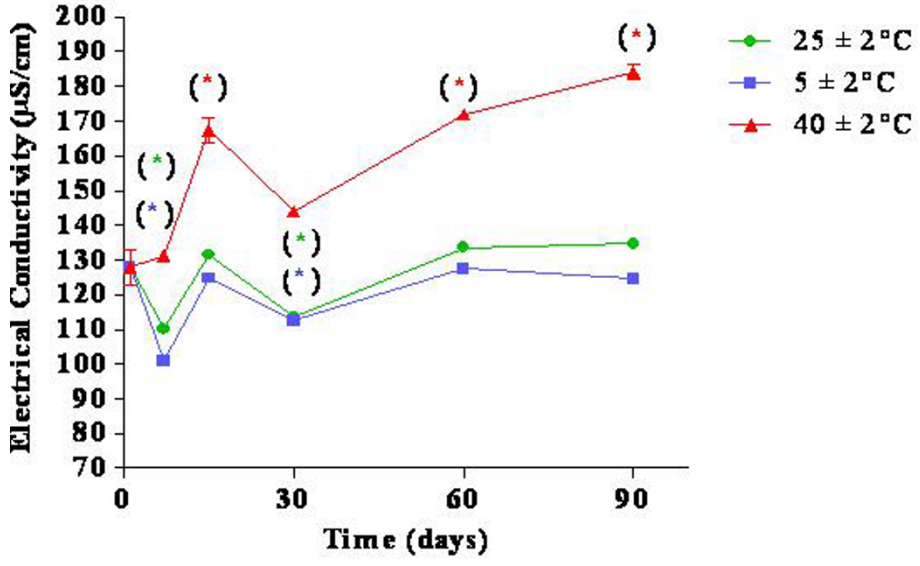

Figure 5 Electrical conductivity of nanoemulsions over time under different storage conditions.

Table 3 Scores and assessments of irritation potential of nanoemulsions and surfactant solution tested in the chorioallantoic membrane test

Formulation	Cumulative Score (average)	Assessment
Nanoemulsion[1]	0	Practically none
Surfactant Solution[2]	5	Moderate
Positive control[3]	15	Strong
Negative control[4]	0	Practically none

[1]10.00% of rice bran oil, 5.00% of sorbitan oleate, 5.00% of PEG-30 castor oil, 0.05% of BHT antioxidant, 0.50% of preservative and 79.45% of distilled water

[2]5.00% of sorbitan oleate, 5.00% of PEG-30 castor oil and 90.00% of distilled water

[3]10% of SLS and 90% of distilled water

[4]Saline solution

the HET-CAM test. This nanoemulsion formulation was applied to volunteers with either normal or affected skin (atopic dermatitis or psoriasis).

Moisturising activity

The moisturising activity of the *stratum corneum* is measured by skin capacitance. It is also an important tool in evaluating healthy and diseased skin such as patients with atopic dermatitis or psoriasis [34-36]. The moisturising variance in healthy volunteers increased both 30 and 60 minutes after nanoemulsion application and then decreased over the remainder of the study. Volunteers with atopic dermatitis or psoriasis showed increased moisturising variance in the first 30 minutes and maintained this increase up to 90 minutes after application. Then, the moisturising capacitance decreased after 90 minutes until the end of test (Figure 6). Skin affected by atopic dermatitis or psoriasis had a lower basal hydration value compared with healthy skin. People with these drying skin conditions have increased dryness in their skin outside of the regions with lesions [37-39]. The rice bran oil nanoemulsion increased the moisturising variance by about 38% in normal skin volunteers and by 30% in volunteers with atopic dermatitis or psoriasis, which is a satisfactory result because a high-quality commercial moisturiser only increased skin hydration by about 20% after 14 days of application [40]. These improved effects may be caused by the nanoemulsion droplets adhering to the skin and forming a dense film that inhibits water evaporation from the skin [41].

The rice bran oil nanoemulsion significantly increased the skin hydration in volunteers suffering from atopic dermatitis and psoriasis. Although the skin hydration measurements should be conducted over a longer period of time, 8 - 24 h, this study indicates that the nanoemulsion may provide long-term skin hydration.

Oily Skin

The oiliness values of nanoemulsion-treated skin increased considerably 30 minutes after treatment and then decreased in both the healthy and affected skin groups. The increase may be related to the amount of rice bran oil (10%) in the formulation (Figure 7). Cosmetic emulsions form an oily layer on the skin that can protect the lipid barrier, which is desirable in dry skin conditions [42]. Therefore, the oiliness of the nanoemulsion may provide an alternative treatment for psoriasis.

Skin pH determination

Forearm skin testing is standard in most clinical studies of skin and has pH values in the range of 4.2 to 5.9 for both sexes [43]. The pH values of volunteers' skin tested during this study ranged from 4.9 to 5.2 after treatment for both groups. Thus, the pH changes due to the nanoemulsion

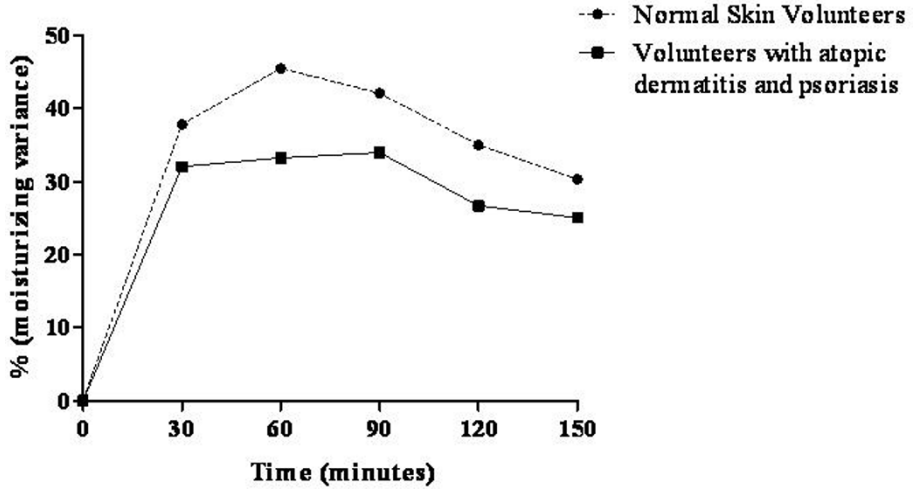

Figure 6 Moisturising variance after nanoemulsion treatment in volunteers with either normal skin or skin affected by atopic dermatitis or psoriasis.

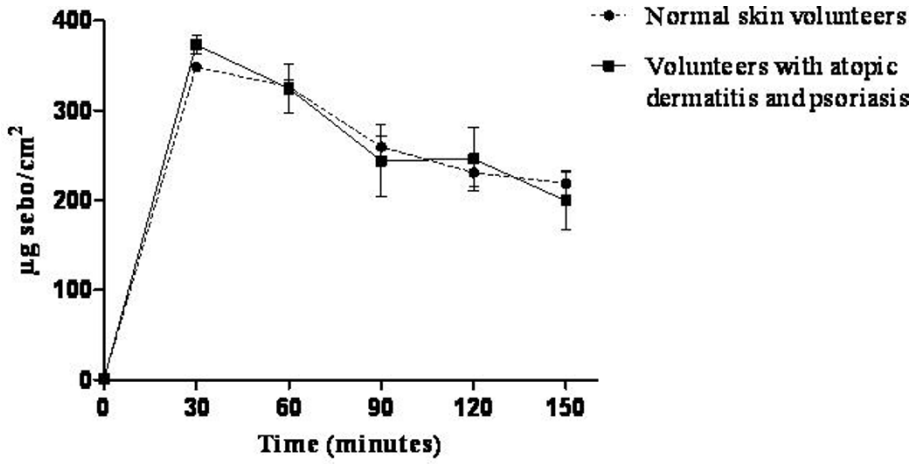

Figure 7 Oiliness of the skin after nanoemulsion treatment in volunteers with either normal skin or skin affected by atopic dermatitis or psoriasis.

were within the accepted pH range for forearm skin found in the literature, so this formulation does not significantly alter the skin pH (Figure 8).

Conclusion

The nanoemulsion developed in this study using the phase diagram method was composed of 10% rice bran oil, 10% surfactants sorbitan oleate/PEG-30 castor oil, 0.05% antioxidants and 0.50% preservatives formulated in distilled water. The nanoemulsion was stable during the period of study and was found to be practically non-irritating in the organotypic HET-CAM model. When applied to the skin of volunteers, the nanoemulsion increased the relative hydration of the skin, the skin oiliness and maintained normal skin pH values. This nanoemulsion could serve as an alternative treatment for skin diseases such as atopic dermatitis and psoriasis.

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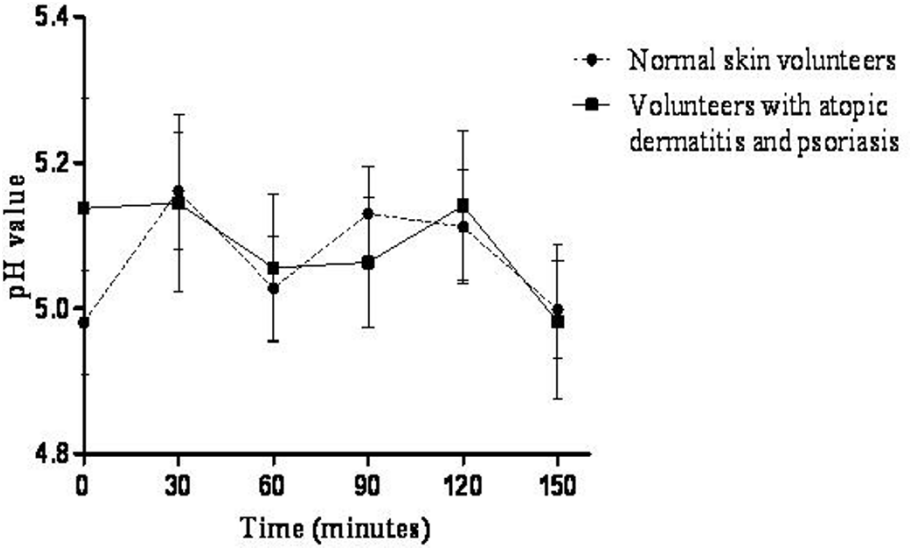

Figure 8 Skin pH value after nanoemulsion treatment in volunteers with normal skin or skin affected by atopic dermatitis or psoriasis.

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Authors' contributions

DSB selected the nanoemulsion composition through the phase diagram study. JB and DSB contributed to the stability study of formulation. NRM and DSB performed the irritant test in the organotypic model - HET-CAM. TAP and GSV performed the *in vivo* assessment. PARF and NRM guided the studies. The manuscript has been read and approved by all the authors.

Competing interests

The authors declare that they have no competing interests.

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EXHIBIT 2

Clinical Pharmacology: Advances and Applications

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REVIEW

Skin models for the testing of transdermal drugs

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Abstract: The assessment of percutaneous permeation of molecules is a key step in the evaluation of dermal or transdermal delivery systems. If the drugs are intended for delivery to humans, the most appropriate setting in which to do the assessment is the in vivo human. However, this may not be possible for ethical, practical, or economic reasons, particularly in the early phases of development. It is thus necessary to find alternative methods using accessible and reproducible surrogates for in vivo human skin. A range of models has been developed, including ex vivo human skin, usually obtained from cadavers or plastic surgery patients, ex vivo animal skin, and artificial or reconstructed skin models. Increasingly, largely driven by regulatory authorities and industry, there is a focus on developing standardized techniques and protocols. With this comes the need to demonstrate that the surrogate models produce results that correlate with those from in vivo human studies and that they can be used to show bioequivalence of different topical products. This review discusses the alternative skin models that have been developed as surrogates for normal and diseased skin and examines the concepts of using model systems for in vitro–in vivo correlation and the demonstration of bioequivalence.

Keywords: percutaneous permeation, dermal delivery, transdermal, bioequivalence, ex vivo skin models, reconstructed skin

Introduction

The skin is a major physical, immunological, and sensory barrier to our environment. While it has long been used as a portal for drug delivery, it is a formidable barrier that requires appropriate technology for successful delivery. It is particularly effective in preventing large (ie, molecular weight >500) or polar molecules from entering the body. It is also a heterogeneous organ, with several delivery routes and sites that could be targeted for desirable pharmacological and immune responses. A key challenge is to deliver to the target site sufficient quantities of the drugs, peptides, vaccines, and dyes that are mainly larger and polar to achieve these responses. This may require the design of a specific chemical or physical delivery system to enhance the permeation of the active substance.

The assessment of percutaneous permeation is key to the successful development of new formulations intended for human use. It is also an important quality-control measure to ensure batch-to-batch uniformity in the pharmaceutical industry.[1] Clinical end-point bioequivalence studies have generally been used for bioequivalence assessments of locally acting products. However, this is not the most feasible approach, due to the high costs involved, as well as the lack of sensitivity in highlighting formulation differences. Alternative methods for evaluating product performance include a

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range of models. More commonly used models to conduct skin-permeation studies are ex vivo human or animal skin. Through the standardization of protocols and techniques, the available skin models can be useful as surrogate models for in vivo human skin to evaluate the bioequivalence of topical products.

This review discusses the alternative skin models that have been developed as surrogates for normal and diseased skin, and examines the concepts of using model systems for in vitro–in vivo correlation (IVIVC) and the demonstration of bioequivalence. Table 1 lists a range of appropriate skin models.

Human skin structure and function

A comprehensive review of the structure and function of skin can be found in Monteiro-Riviere.[2] The skin accounts for approximately 16% of human body weight, with a surface area of approximately 2 m^2 in adults.[3,4] It provides a physical barrier to the environment, maintains homeostasis by limiting the loss of water, electrolytes, and heat, and protects against microorganisms, toxic agents, and ultraviolet radiation.[5] There are three basic layers: the epidermis, the dermis, and the subcutaneous layer. Hair, nails, sebaceous glands, and sweat glands (apocrine and eccrine) are considered to be skin derivatives or appendages. Even though it is structurally continuous throughout the body, skin varies in thickness according to the age of the individual and the anatomical site.

The epidermal layer is formed from squamous epithelium and is subdivided into separate layers, according to the degree of keratinization of the cells. The layers of the epidermis from the bottom to the surface are stratum basale (basal cell layer), the stratum spinosum (spinous or prickle-cell layer), the stratum granulosum (granular cell layer), and the stratum corneum (SC; horny layer) (Figure 1).[6]

The outermost layer of the epidermis, the SC, consists of denucleated, nonliving, flattened cells called corneocytes. There are ten to 25 layers of stacked corneocytes, which are nonhydrated cells lying parallel to the skin surface.[5] The SC layers are united by SC lipid bilayers assembled into a "brick and mortar" arrangement.[7]

Below the SC, the remainder of the epidermis is viable tissue, called viable epidermis, containing nucleated cells called keratinocytes. The viable epidermis is a region for drug binding, metabolism, active transport, and surveillance. In addition to keratinocytes, it contains melanocytes (dendritic cells found on the basement membrane and in the basal layer), Merkel cells (functioning as mechanoreceptors involved in mediation of touch responses, found in the basal region), and Langerhans cells (dendritic cells playing a key role in protective immune function, present mainly in the stratum spinosum).[2]

The viable epidermis is separated from the dermis at the dermal–epidermal junction. The dermis is rich in collagen. The subcutaneous (hypodermis) layer is the deepest layer of the skin and is formed from loose connective tissue and fat (50% of the body fat), which may be more than 3 cm thick on the abdomen. The dermis and subcutaneous layers contain blood vessels, lymphatics, and nerve cells, in addition to skin appendages.[8]

Table 1 Skin models

Model	Advantages	Disadvantages
Human skin		
In vivo	Gold standard	Often precluded for ethical and practical reasons
Ex vivo skin	Best surrogate for in vivo humans	Not readily available, variability
Animal skin		
In vivo	Reasonably easy to obtain animals, can be scaled up to humans, hairless species available	Pigs: similar barrier to humans, but difficult to handle Rodents: different barrier properties from humans
In vivo chimeric model	Human skin xenografts on mice allows testing on living human skin	Technically difficult
Ex vivo skin	Easy to obtain	Different barrier properties, variability
Artificial membranes		
Simple polymeric models	Useful for studying basic diffusion mechanisms, consistent and homogenous	Not representative of human skin
Lipid-based models	Useful for screening	Not representative of human skin
Reconstructed skin models		
Reconstructed human epidermis	Built-in barrier properties	Usually more permeable than human skin
Living skin equivalents	Can be engineered to include a range of normal or disease features	Usually more permeable than human skin

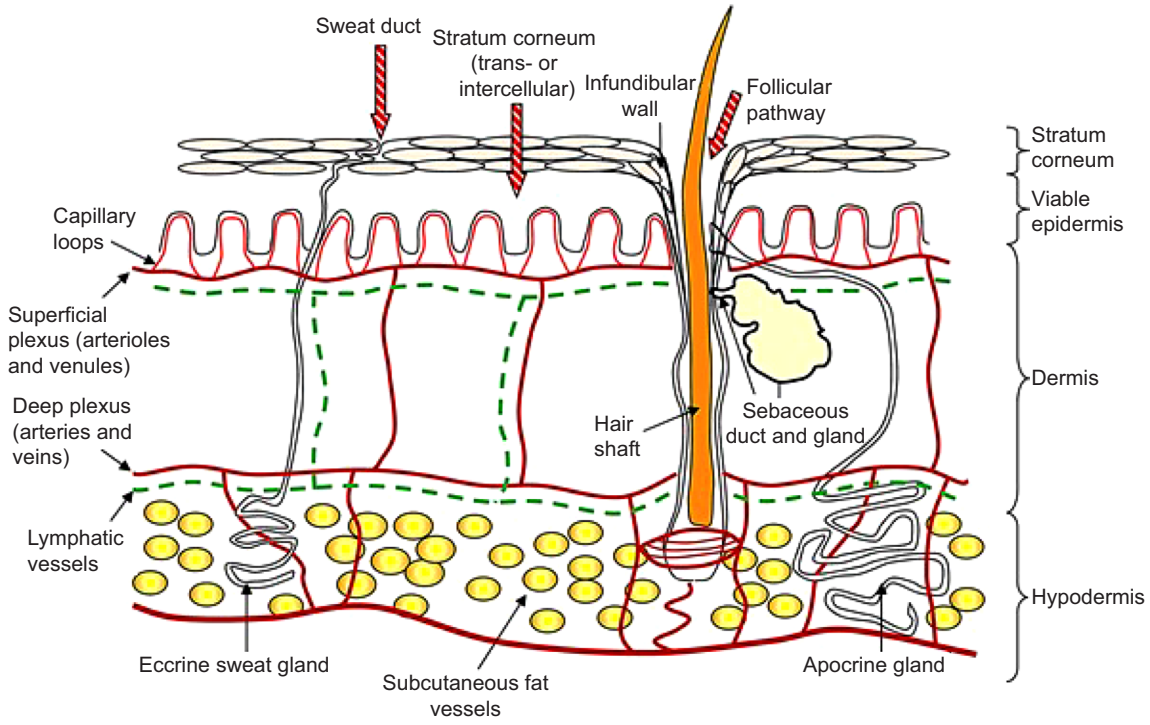

Figure 1 Structure of the skin.

The SC acts as the primary skin barrier, with the essential functions of protecting the body from the surrounding environment, providing an efficient obstacle to the permeation of exogenous molecules[8] and microorganisms[9] and maintaining homeostasis by preventing excessive loss of water.[10] The surveillance, metabolic, and transport processes located in the deeper skin layers[11,12] also contribute to the protective functions of the skin.

Models used to evaluate dermal absorption

Ex vivo human skin models

Measurement of dermal absorption for the purpose of targeted skin delivery, systemic delivery, or toxicological assessment should be done under the correct conditions, ideally using the gold-standard experimental model: in vivo human skin.[13] However, this is not always possible, due to the high cost of human trials and concerns over applying substances or materials with potentially toxic effects. As well, in vivo responses may be difficult to measure and interpret and subject to significant variability. Alternative methods are needed to derive data that are reproducible and reliable and which provide a meaningful prediction of the in vivo human situation.[14] There is a large body of work based on ex vivo human skin, and as we shall discuss in more detail later, some success has been achieved in correlating in vitro and in vivo dermal absorption, often driven by regulators seeking a standard, robust assessment method.[15,16]

Excised human skin is most commonly obtained from plastic surgery or cadavers, and in both cases, appropriate ethical approval is required to use the tissue. Abdominal, breast, or back skin is most convenient, due to the large areas that may be available. There are considerable differences in skin absorption across different body sites,[17] attributed to such factors as differences in SC thickness,[18] hydration,[19,20] and lipid composition.[21] Clearly, this needs to be recognized when designing studies.

As noted earlier, the SC represents the main barrier to penetration of exogenous substances into the skin,[8] as well as controlling the loss of water from inside the body.[10] It is believed that skin may be stored for up to 6 months without loss of SC barrier function,[22] particularly if 10% glycerol is used as a preservative.[23] Nielsen et al also found little effect of freezing at –20°C for 3 weeks, with or without polyethylene glycol as a preservative, but significant damage with storage at –80°C.[24] On the contrary, other evidence suggests significant loss of barrier function, causing increased skin permeation, with frozen storage of animal skin.[25,26] Barbero and Frasch concluded that carefully handled frozen human skin was suitable for testing the passive permeation of chemicals, when skin viability and metabolic activity were not being investigated.[23] Cellular

viability and metabolic activity within the human epidermis are likely to be reduced by frozen storage or heat.[27,28] Our unpublished results from an MTT assay showed a complete elimination of epidermal viability following heat separation by the method of Kligman and Christophers.[29] For studies requiring the presence of viable epidermal tissue, such as imaging of endogenous skin autofluorescence by multiphoton tomography,[30] or investigations of skin metabolism,[28] fresh tissue is required.

Several different membrane types may be prepared from ex vivo human skin for use in permeation experiments. "Full-thickness skin" is prepared by removal of connective tissue and subcutaneous fat and consists of all layers down to and including the dermis.[31] To reduce variability while retaining significant dermal thickness, full-thickness skin may be cut to approximately 500–750 µm with a dermatome.[32] However, the presence of the hydrated dermis may introduce an additional, artificial barrier to permeation, particularly for more lipophilic molecules.

In contrast to the in vivo situation, where capillary circulation rapidly clears penetrated molecules, full-thickness or dermatomed skin mounted in a diffusion cell represents a situation analogous to vasoconstriction.[33] Consequently, the use of a membrane consisting of only the SC and the viable epidermis may be preferred. According to Cross and Roberts, this membrane represents a situation of "infinite dilatation", since all material making its way past the SC barrier is immediately available to the receptor solution.[33] Atrux-Tallau et al found that dermatomed human skin and heat-separated epidermal membranes gave the same flux for caffeine, a hydrophilic compound.[34] Results from our group where steroids were applied to epidermal membranes, full-thickness skin, and isolated dermis showed that there was a minimal effect of increasing lipophilicity on epidermal maximum flux and a trend toward decreased dermal penetration rates.[35]

Epidermal membranes are most commonly prepared by immersing full-thickness skin in hot water (~60°C) for approximately 1 minute.[29] Other techniques designed to separate the membrane at the dermal–epidermal junction include chemical treatments with ethylenediaminetetraacetic acid, ammonia, and enzymes.[32] Some researchers have used the isolated SC for permeation experiments[36] and for desorption studies designed to study SC heterogeneity,[37] and the SC reservoir for water and other substances.[38] The SC is prepared by enzymatic methods, usually by incubation with aqueous trypsin solution, after which the digested epidermal material is rinsed and wiped off.[39]

Ex vivo human skin: use in bioequivalence studies

For the majority of topical drug products, comparative clinical end-point studies are used to demonstrate bioequivalence to a reference drug. While this provides a direct in vivo assessment, it is also associated with a number of challenges. Clinical end points are associated with high variability (intra-subject) and low sensitivity (drug-related), which makes such studies less reliable and less efficient. The other clinical alternative is to use pharmacokinetic studies to demonstrate bioequivalence for topical products, but this is limited to particular cases where significant systemic absorption of the drug occurs. Recently, the use of techniques including in vitro permeation testing (IVPT), in vivo tape stripping, or dermatopharmacokinetics, and in vivo microdialysis or microperfusion, has been advocated for testing bioequivalence.[40]

IVPT using human dermatomed skin mounted in diffusion cells is increasingly seen as a suitable tool for demonstrating bioequivalence of topical dosage forms.[40] Indeed, the generation of such data has been encouraged by regulatory agencies, such as the European Medicines Agency, the US Environmental Protection Agency, and the US Food and Drug Administration (FDA). Their utility is grounded on substantial evidence that 1) there is a good correlation between the in vitro and in vivo rates and extents of human skin absorption of a number of different substances and 2) there is good agreement on the bioequivalence of topical products seen with IVPT and in vivo clinical studies.

Currently, however, there are no approved protocols for carrying out IVPT studies. Franz et al pointed out that the demonstration of valid IVIVC is greatly dependent upon the protocols used, and they recommended that the in vitro and in vivo protocols followed should be as closely harmonized as possible to maximize the chance of achieving a good correlation.[15,41] The work on which this conclusion was based was an analysis of historical literature data that was available to the researchers, and despite the wide variation in the way in which it was collected, their conclusion was that there was compelling evidence that it was possible to correlate IVPT data with human in vivo skin-absorption data. Others who have demonstrated good IVIVC include Hadgraft et al, who compared in vitro and in vivo delivery from nitroglycerin patches,[42] and more recently Yang et al, who compared their own IVPT data with literature reports of in vivo estradiol delivery from patches.[16]

While the use of IVPT for bioequivalence has only recently been formalized, the design of in vitro permeation tests has been subject to consideration and validation for many years. In 1987, the FDA published a report on the

important factors to be considered, which included the membrane type (dermatomed skin or heat-separated epidermis?), the receptor fluid, the cell design (static or flow-through?), application (finite or infinite dose?), and temperature.[43]

The in vivo dermatopharmacokinetic (DPK) method uses tape stripping to remove SC layers. The FDA has investigated the possibility of introducing a DPK method for evaluating bioavailability and/or bioequivalence of topical dermatological drug products.[44,45] In the DPK method, it is assumed that 1) in normal circumstances, the SC is the rate-determining barrier to percutaneous absorption, 2) the SC concentration of the drug is related to the amount that diffuses into the underlying viable epidermis, and 3) SC drug levels are more useful and relevant for assessing local, dermatological efficacy than plasma concentrations.[46] It is also possible to deduce partitioning and diffusion parameters that characterize the absorption process and which can subsequently be used to predict an entire absorption profile from a single short-contact-duration experiment.[44] The technique is very operator-dependent, and care needs to be taken to apply and remove the tapes reproducibly. The success of the method is equally dependent on the development of sensitive analytical methods to quantify the amount of drug in the tapes.

Microdialysis involves the insertion of an ultrathin hollow fiber as a probe into the dermis. The probe is semipermeable and perfused with sterile buffer using a microdialysis pump. This involves the exchange of the small diffusible molecules from the extracellular fluid into the probe and vice versa. This method is used to determine the concentration of the unbound drug or biomarkers at the site to establish the concentration-versus-time profile of the applied compound. There are several issues associated with microdialysis. Probe insertion in the skin can lead to inflammatory responses, as may interactions of the perfusing buffer with the tissue.[47,48] Recovery is low for highly lipophilic molecules, which may be resolved to some extent by using albumin, cyclodextrins, and cosolvents such as ethanol and dimethyl sulfoxide in the buffer,[49] while highly protein-bound molecules may be difficult to detect, due to binding to the probe material. A major disadvantage of the method is the intrasubject variability.[50] The newer technique of dermal open-flow microperfusion (dOFM) differs from microdialysis, in that it gives continuous, membrane-free (ie, unfiltered) access to dermal fluid.[51] Like microdialysis, dOFM provides a direct estimate of the time course of delivery of the permeant near its site of application. Because of the lack of interaction with a membrane in dOFM, it can be used for a wider range of compounds than microdialysis.[40] The technical difficulty of microdialysis and dOFM means that significant operator expertise is required, and as such they are generally only available in a research setting.

Ex vivo animal skin models

The assessment of percutaneous absorption of molecules is an important step in the evaluation of any topical drug-delivery system or formulation. As we have already noted, if the dosage form is to be used in humans, the most relevant skin-absorption data should come from in vivo human studies. However, such studies are generally not feasible during the initial development of a novel pharmaceutical dosage form. Moreover, ex vivo human skin may not be readily available, and so researchers have relied on animal studies for much of the experimental data. This creates a major challenge in correlating results from ex vivo animal experiments with ex vivo and in vivo human studies for prediction of human percutaneous absorption.

A wide range of animal models has been used as alternatives to human skin to evaluate percutaneous permeation of substances. These include pig, mouse, rat, guinea pig, and snake models. Porcine (pig) skin is histologically similar to human skin,[52,53] with a comparable SC thickness of 21–26 μm.[54,55] In addition, the average hair-follicle density in porcine ear skin is 20/cm^2 compared to 14–32/cm^2 in human forehead skin.[54] As well as being similar to human skin, porcine ear skin is also convenient to obtain and has been widely used in skin-permeation studies.[56]

The SC lipids are known to be important regulators of skin permeability. With this in mind, the conformational disordering and lateral packing of lipids in isolated porcine and human SC were compared using Fourier-transform infrared spectroscopy. The SC of both species differ markedly, with porcine SC lipids being arranged predominantly in a hexagonal lattice, while lipids in human SC were predominantly packed in the denser orthorhombic lattice.[57] In human as well as porcine SC, the main lipid classes are ceramide, cholesterol, and free fatty acid, and these lipid classes are present in an approximately equimolar ratio.[58] However, the compositions of free fatty acid and ceramide in the two species are different.

In a range of studies using both lipophilic[59,60] and hydrophilic[59,61] permeants, the permeability of pig skin was found to be similar to that of human skin, but to differ to a greater extent from dog[61] or rodent skin.[59,61] Sato et al attributed the similarity in permeability to the similar SC lipids, barrier thickness, and morphological aspects of pig and human skin.[61] Nicoli et al further investigated the differences

between pig skin and rabbit ear skin, finding that although they had similar SC thicknesses, pig skin was four to seven times more permeable to hydrophilic compounds than rabbit ear skin, most likely due to its different SC lipid composition.[62] The relationship between permeability and SC lipids is analogous to early findings by Lampe et al,[63] who showed the total lipid-weight percentage at various human body sites (face > abdomen > leg > plantar SC) was inversely proportional to the relative permeability of skin reported for those sites by Scheuplein and Blank.[64] Caussin et al also reported the similarity in SC lipid composition, as well as in lamellar organization, between pigs and humans.[63,65] Interestingly, however, they also saw a substantial difference in lateral packing between the two species. As with human skin, permeation behavior was found to correlate with barrier function, as measured by transepidermal water loss in a study by Sekkat et al, who applied caffeine, lidocaine, and phenobarbital to tape-stripped pig skin.[66]

Skin of rodents (mice, rat, and guinea pigs) is the most commonly used in in vitro percutaneous permeation studies, due to its availability, their small size, and relatively low cost. There are different hairless strains of each species that are reported to mimic the permeation properties of human skin better than the hairy variety.[67] Among rodents, rat skin is most structurally similar to human skin and it is the most frequently used rodent model. A large number of studies comparing permeation through human and rat skin have been carried out, showing that rat skin is generally more permeable than human skin across a range of permeants of different physicochemical properties,[68–72] in some cases with differences of more than an order of magnitude. For example, for compounds with log-*P*-values ranging from 0.7 to 4.5, van Ravenzwaay and Leibold[72] found that mean in vitro permeation flux through rat skin was around elevenfold greater than through human skin, while a similar comparison by Schmook et al[71] found flux increase of 50-fold for the relatively lipophilic molecules hydrocortisone and terbinafine.

Shed snake skin is another interesting membrane that was suggested as a suitable alternative to human skin.[73] This membrane, which can be obtained without killing the animal, has some similarity to human skin, in that it consists of thin, flat squamate cells surrounded by intercellular phospholipids, although it does lack hair follicles. Rigg and Barry compared permeation of fluorouracil (5-FU) through dermatomed human abdominal skin and shed skin from two snake species.[74] The permeability coefficients were similar between human and dorsal and ventral skin of one snake species, whereas there was a 30-fold increase in dorsal skin from *Elaphe* (*Pantherophis*) *obsoleta*. These authors found no changes in 5-FU permeability in human or snake skin after acetone pretreatment, whereas Megrab et al[75] found differential responses in human and dorsal snake skin with vehicles containing different ethanol concentrations. Apart from possible interspecies differences, it is likely that solvent effects in snake skin are influenced by both the lower water content[75] and the nature of the intercellular lipids. While snake skin may be a reasonable model for human skin, it is not readily available, and doubts must exist over the quality and consistency. As Rigg and Barry noted, "[. . .] if at all possible, investigative problems should not be made more complex by selection of an animal tissue to represent human skin".[74]

It may be useful, particularly in the interpretation of dermal absorption for human risk assessment, to predict human in vivo dermal absorption from known in vitro human, in vivo animal, and in vitro animal data, the so-called triple-pack approach.[76] The animal in question is normally considered to be the rat. Human in vivo dermal absorption may be derived by the equation:

$$\text{in vivo human absorption} = \text{in vivo rat absorption} \times (\text{in vitro human absorption} / \text{in vitro rat absorption}) \quad (1)$$

Here, it is assumed that 1) the factor between in vitro and in vivo dermal absorption is the same for rats and humans and 2) the factor between rat and human skin absorption is the same in vitro and in vivo, despite the morphological species differences.

Artificial and reconstructed skin models

Artificial and reconstructed skin models are useful tools in specific circumstances, driven by the need to find convenient, reproducible alternatives to in vivo and ex vivo tests with human and animal skin. The artificial skin models range from simple homogeneous polymer materials, such as poly(dimethoxysilane) or silicone membranes through to lipid-based parallel artificial membrane-permeability assay (PAMPA) or phospholipid vesicle-based permeation-assay membranes,[77] with the latter material designed to mimic the SC. By eliminating the complexity of human or animal skin, the simple homogeneous materials are particularly useful for studying the basic mechanisms controlling passive transport though a membrane.[78–80] The main advantage they have in this regard is their relative reproducibility due to their simple standardized construction. However, they are not intended to represent, nor are they capable of, representing the multitude of in vivo skin properties.[81]

The PAMPA can be used for rapid screening of passive transport.[82] The PAMPA assay is conducted in a 96-well filter plate coated with a liquid artificial membrane to separate two compartments: one containing a buffer solution of compounds to be tested (donor compartment) and the other containing an initial fresh buffer solution (acceptor compartment). Significant correlations with gastrointestinal absorption in humans were seen with PAMPA using filters impregnated with a solution of phospholipids or hexadecane.[82] To develop a new artificial membrane to be used in PAMPA for prediction of skin permeation, Ottaviani et al investigated the permeability coefficients of a number of compounds through human skin and the PAMPA-skin artificial membrane comprised of dimethylpolysiloxane (silicone) membranes. They reported a good correlation between the two skin models.[83]

The FDA has encouraged the use of porous synthetic membranes for evaluating the performance of topical products, as they act as a support without posing a rate-limiting barrier.[84] Shah et al from the FDA used different microporous membranes, such as pure cellulose acetate, cellulose, and polysulfone, of similar pore sizes and thicknesses to examine the permeation of hydrocortisone from two commercial creams. They found that the hydrocortisone flux was consistent irrespective of the types of synthetic membrane.[85] Nitroglycerin drug release from commercial ointments was investigated by Wu et al[86] using ten types of commercial synthetic membranes, such as polysulfone, cellulose mixed esters, polytetrafluoroethylene, and polypropylene, with different pore size and thickness. From the results obtained in this study, the synthetic membranes may be classified into two groups: group 1, consisting of polysulfone, acrylic polymer, glass fiber, silicone, and mixed cellulose ester, showed higher drug permeation compared to group 2, which included polytetrafluoroethylene–polyethylene, mixed cellulose ester (of greater thickness), and polypropylene. The effect of membrane types upon ketoprofen drug release from a gel has also been studied. It was found that nylon exhibited the least rate-limiting effects, although it is a thicker synthetic membrane compared to others.[87]

Reconstructed skin models are culture-based, with layers of human cells in culture laid down over a polymeric matrix. This allows different cell types to be incorporated to achieve a structure of the desired composition and complexity. Reconstructed models are generally designed to simulate the epidermis (reconstructed human epidermis [RHE] models) or the full human skin (living skin equivalents [LSEs]).[22,77]

Some reconstructed skin models are produced in-house for particular research purposes, such as drug-candidate or toxicological screening[88] or the assessment of photodamage and photoprotection.[89] In one particular reconstructed model, consisting of layers of human dermal fibroblasts and human epidermal HaCaT cells, there was no change in the permeability coefficients of ibuprofen after freezing the membrane over liquid nitrogen for 24 hours or 6 months. Such a property would make reconstructed membranes attractive for general screening uses. In addition, there are commercially available RHEs (eg, EpiSkin®, SkinEthic®, and EpiDerm®) and LSEs (eg, GraftSkin®, EpiDermFT®, and Pheninon®) that have been suggested as suitable candidates for in vivo and ex vivo skin models in evaluating skin absorption, testing of cosmetic products, and for the toxicological screening of topically applied compounds. A number of studies have compared LSE and HRE models with animal and human skin.[71,90,91] Schmook et al studied salicylic acid, hydrocortisone, clotrimazole, and terbinafine permeation through ex vivo human (dermatomed), porcine, and rat skin, GraftSkin LSE, and SkinEthic RHE.[71] The fluxes and skin accumulation were generally in the order human ≤ porcine < rat < GraftSkin << SkinEthic. Comparing human and pig skin with two RHE models, Schreiber et al[91] found permeation coefficients of caffeine and testosterone were both in the order human < pig < EpiDerm << SkinEthic. Schäfer-Korting et al published an extensive comparison of human epidermal membranes, porcine skin, and three RHE models – EpiDerm, EpiSkin, and SkinEthic – with a series of hydrophilic and lipophilic permeants.[90] Their general conclusions were that the RHE models, particularly SkinEthic, were significantly more permeable than the ex vivo skins, although the ranking of the permeation of the compounds through pig skin and the RHEs mirrored that through human epidermis. Interestingly, they did not observe the expected improvement in reproducibility with the RHEs compared to the ex vivo skin.

As of 2013, reconstructed skin models had received Organization for Economic Co-operation and Development approval for testing of skin corrosion, acute skin irritation, and phototoxicity.[22] None is currently approved for testing of skin absorption. Further work is needed to validate the various models, particularly the LSEs, for this purpose, although they may be useful for in vitro screening. Interestingly, Schäfer-Korting et al concluded that the tested RHEs were applicable to both finite- and infinite-dose studies.[90]

Models for skin diseases

The skin is not only a convenient portal to the systemic circulation but also a logical site of application for treatment

of various localized skin disorders, such as skin cancers, inflammatory illnesses, and damaged skin. As the investigation of disease mechanisms and new therapies is usually difficult or impossible in humans, it is necessary to use alternative methods. Models representing normal or healthy skin are appropriate to test the delivery and targeting of topically applied drugs or other substances, often for the purpose of evaluating the delivery system used. However, models that are designed to mimic the effects of disease states can be used to study the delivery and effects of topical therapies or to gain insight into the molecular mechanisms responsible for particular diseases. In the following sections, we review some of the various animal and artificial models that have been applied to studies of skin diseases. Some recently published studies using animal and reconstructed human skin models for the study of skin diseases, including their use in therapeutic screening, are summarized in Tables 2 and 3.

Ex vivo animal models for skin diseases

A plethora of in vivo animal models employing fish, guinea pigs, mice, rats, rabbits, and pigs have been developed to mimic human skin diseases. Some recent reviews have focused on the most widely studied areas of melanoma,[92] atopic dermatitis,[93] and psoriasis.[94] Other applications include skin infections (eg, acne, viral infections), damaged skin (eg, wounding, photo-damaged skin), hair disorders (eg, different types of alopecia), and skin cancers, such as basal and squamous cell carcinomas. A significant number of models use genetically engineered mice, due to the fact that many human skin diseases are cause by gene mutations.[95] These animal models have been extensively used for the understanding of disease mechanisms and to a lesser extent for the clinical evaluation of drug candidates. For example, epidermal VEGF-knockout mice were used to identify a specific role for epidermal VEGF in the maintenance of epidermal permeability-barrier homeostasis and pointed to the disruption of VEGF pathways in the development of psoriasis.[96] In very recent work by Rossbach et al,[97] histamine H_4 receptor (H_4R)-knockout mice showed significant reductions in ovalbumin-induced skin lesions analogous to those caused by atopic dermatitis. Their findings suggested that H_4R could be a new therapeutic target in allergic skin diseases like atopic dermatitis.

In addition to melanoma, mouse models have been used particularly for the other common skin cancers, squamous cell carcinoma,[98–103] and basal cell carcinoma.[104–106] An overview of animal models for a wide range of skin conditions, with an emphasis on their application to drug discovery, has been published by Avci et al.[107]

Of particular interest are the chimeric models, in which living human skin is grafted on to the skin of severe combined immunodeficient (SCID) mice. In this way, responses or treatments can be studied in living human skin. For example, targeted Kv1.3-cell immunotherapy was shown to be effective in reducing human epidermal thickness and the number of $CD3^+$ lymphocytes in an SCID mouse–human psoriatic skin xenograft model, leading the authors to propose the investigated therapy for treatment of psoriasis and possibly other inflammatory skin conditions.[108] Similar investigative work in psoriasis used the SCID mouse–human psoriatic skin xenograft model to identify a role for Hsp90 in signaling pathways that are upregulated in psoriasis. Mice treated orally with the Hsp90 inhibitor Debio 0932 showed a reduction in xenograft epidermal thickness.

The xenograft model has also been used for investigation of cancer targets and therapies. Targeted oral[109] or intravenous[110] treatments in SCID mouse–human melanoma xenografts caused significant reductions in tumor proliferation and size in BRAF- and $ALDH^+$-specific melanomas.[109,110]

Reconstructed skin models for diseases

Today, there are increasing regulatory restrictions on the use of animals, and the availability of excised human diseased skin is limited. For these reasons and following the advances in tissue engineering, the development of artificial in vitro human skin models to mimic both healthy and diseased skin has intensified. Another important benefit of using artificial skin models is that they allow the incorporation of specific disease characteristics in a controlled and relatively reproducible manner. In vitro models have been developed for a wide range of skin diseases, such as inflammatory disorders, fungal infections, skin cancer, photodamaged skin, and wounding. A general review has recently been published by Küchler et al.[22] The models are generally developed in-house by researchers, with the goals of understanding disease mechanisms and progression, or less commonly to use as screening tools for the assessment of therapeutic modalities. A major challenge in the use of these models is to assess whether they are relevant to and predictable of the in vivo situation.

Inflammatory and autoimmune diseases for which artificial skin models have been developed include psoriasis,[111,112] atopic dermatitis,[113,114] and eczematous dermatitis.[115] In most cases, the specific pathway leading to expression of the disease state was induced by suitable interventions, such as stimulation by psoriasis-associated cytokines,[112] or in the

Table 2 In vivo animal disease models

Disease model	Characteristics	Drug delivered	Reference
Psoriasis mouse model	Epidermal VEGF-knockout mice used to identify specific role for VEGF in permeability-barrier maintenance		Elias et al[96]
Atopic dermatitis mouse model	Histamine H_4 (H_4R)-knockout mice used to show H_4R modulates inflammation in a chronic allergic dermatitis setting	H_4R antagonists partially mimicked effects of H_4R knockout	Rossbach et al[97]
Dermatophytosis guinea-pig model	Tinea corporis induced by application of *Trichophyton mentagrophytes* TIMM1189 inoculum on dorsal skin	Luliconazole	Koga et al[121]
Irritant dermatitis hairless guinea-pig model	Induced by daily exposure for 4 days to sodium lauryl sulfate	Basic, carbomer, isopropyl palmitate, glycerol, canola oil, and bisabolol creams	Andersen et al[122]
Squamous cell carcinoma mouse model	Dorsal UVB irradiation (minimal erythema dose) of SKH1 hairless mice	Diclofenac (anti-inflammatory COX2 inhibitor) as preventive drug	Burns et al[98]
	UV-induced T7 SCC line subcutaneously injected in the back of SKH1 hairless mice	Ingenol mebutate	Cozzi et al[99]
	UV radiation of SKH1 hairless mice	17AAG (heat-shock protein 90 inhibitor) alone or in conjunction with UVR treatments	Singh et al[100]
	Human skin SCC cell line SRB12-p9 subcutaneously injected into severe combined immunodeficiency (SCID) mice	Curcumin	Sonavane et al[101]
	Two-stage skin-carcinogenesis model in FVB/N mice: 1) topical treatment with carcinogen agent (DMBA), 2) tumor-promoter treatment (TPA), and 3) oral dose with a BRAF inhibitor (PLX4270)	5-Fluorouracil (5-FU)	Viros et al[102]
	Dorsal UVB irradiation (minimal erythema dose) of SKH1 hairless mice	5-Aminolevulinic acid (5-ALA) in conjunction with photodynamic therapy (PDT)	Wang et al[103]
Basal cell carcinoma mouse model	Neonatally irradiated $Ptch1^{+/-}$ mice as a model of Hedgehog (Hh)-signaling pathway-dependent tumors	MK-4101, potent inhibitor of Hh-signaling pathway, had robust antitumor activity	Filocamo et al[104]
	BCC mouse model used to identify molecular mechanisms regulated by Sox9, leading to tumour initiation and invasion		Larsimont et al[105]
	Induced in *PTCH*-knockout mice by 1) treatment by tamoxifen administered intraperitoneally and 2) ionizing irradiation	CUR61414 (an inhibitor of the Hh signal-transduction molecule Smoothened)	Tang et al[106]
Melanoma mouse model	Tumor spheroid of B16/F0 melanoma cells subcutaneously inoculated in the auricle of NMRINu/Nu or C57/BL6 mice	Bortezomib (inhibitor of the 26S proteasome)	Schröder et al[123]
	B16BL6 melanoma cells subcutaneously injected into the notum of C57BL/l mice	Curcumin	Chen et al[124]
	B16 melanoma cells subcutaneously injected in the hip of BALB/c nude mice	Mitoxantrone (DNA-synthesis and -transcription inhibitor)	Yu et al[125]
Human–SCID mouse xenograft model: psoriasis	SCID mouse–human psoriasis skin model used for targeted topical immunotherapy	Kv1.3 channel blocker PAP-1	Kundu-Raychaudhuri et al[108]
Human–SCID mouse xenograft model: melanoma	Identified an intronic mutation as molecular basis for a RNA splicing-mediated RAF inhibitor-resistance mechanism and a pre-mRNA-splicing interference as a potential therapeutic strategy for drug resistance in BRAF melanoma	Vemurafenib, potent RAF-kinase inhibitor	Salton et al[109]
Human–SCID mouse xenograft model: melanoma	Effects of chemical inhibition of ALDH1 on the response of human melanoma xenografts to chemotherapy and the effects of ALDH1A1 RNA silencing on melanoma growth and metastasis; ALDH1 inhibition may be useful in melanoma treatment	ALDH1 inhibitors (eg, diethylaminobenzaldehyde) added to dacarbazine chemotherapy	Yue et al[110]

Abbreviations: UVB, ultraviolet B; SCC, squamous cell carcinoma; DMBA, 7,12-dimethylbenz-(*a*)anthracene; BCC, basal cell carcinoma; mRNA, messenger RNA; BRAF, v-Raf murine sarcoma viral oncogene homolog B; RAF, a serine/threonine protein kinase product of BRAF gene; ALDH, aldehyde dehydrogenase; SCID, severe combined immunodeficient; VEGF, vascular endothelial growth factor.

generation of atopic dermatitis by downregulation of filaggrin[113] or treatment with an inflammatory cocktail.[114] Some of the models were developed as potential screening tools for drugs to treat the expressed disease states.[112,114]

Skin-cancer models were constructed by incorporating various tumor entities within the three-dimensional (3-D) matrix, including cultured melanoma[116] cells, an A375 metastatic melanoma cell line,[117] and melanoma-tumor

Table 3 Reconstructed skin-disease models

Disease model	Characteristics	Drug delivered	Reference
Psoriasis	Full-thickness skin model closely resembling in vivo epidermal architecture used to identify IL-17-responsive genes in psoriasis	Ixekizumab (IL-17 antagonist)	Chiricozzi et al[111]
	Human psoriatic skin equivalents used to study cytokine-induced gene expression	Retinoic acid, cyclosporine A	Tjabringa et al[112]
Atopic dermatitis	3-D reconstructed human epidermis model used to show filaggrin downregulation in the epidermis of atopic patients, either acquired or innate, may be directly responsible for some of the disease-related alterations	Inflammatory cocktail (polyinosinic–polycytidylic acid, TNFα, IL-4, and IL-13)	Pendaries et al[113]
Atopic dermatitis	Compromised reconstructed epidermis mimicking AD-related inflammation in vitro		Rouaud-Tinguely et al[114]
3-D model of dermatitis	Human foreskin fibroblasts HaCaT cells Memory-effector ($CD45RO^+$) T cells Scaffold material: tat tail collagen type I, fibronectin	Dexamethasone and tacrolimus	Engelhart et al[115]
3-D model of melanoma	3-D human skin reconstruct model incorporating melanocytic cells		Li et al[116]
3-D skin-reconstruction model of metastatic melanoma	Human malignant melanoma cells (A375) Normal human-derived epidermal keratinocytes (NHEKs) Normal human-derived dermal fibroblasts (NHDFs) Scaffold material: collagen type I	Roscovitine (cyclin-dependent kinase inhibitor)	Mohapatra et al[117]
3-D organotypic skin-melanoma spheroid model	Human melanoma cell lines SBCL2 (RGP), WM-115 (VGP), and 451-LU (MM) Human primary keratinocytes Human primary fibroblasts Scaffold material: rat tail collagen type I	TRAIL + ultraviolet B radiation TRAIL + cisplatin	Vörsmann et al[118]
3-D model of human cutaneous squamous cell carcinoma	Primary NHEKs Primary NHDFs, SCC12B2 and SCC13 cell lines Scaffold material: rat tail collagen type I Pretreatment with EGF	Erlotinib (tyrosine-kinase inhibitor)	Commandeur et al[119]
3-D model of scleroderma fibrosis	Model useful for testing in vivo the progression of scleroderma and to screen for antifibrotic drugs	Nilotinib, a tyrosine kinase inhibitor; human monoclonal anti-PDGFR antibodies	Luchetti et al[120]

Abbreviations: IL, interleukin; TNFα, tumor necrosis factor alpha; 3-D, three dimensional; NHEKS, Normal human-derived epidermal keratinocytes; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; SBCL-2 (RGP), an early radial growth phase cell line; WM-115 (VGP), a vertical growth phase cell line; MM, metastatic melanoma; NHDF, normal human-derived dermal fibroblasts.

spheroids,[118] as well as various cutaneous squamous cell carcinoma cell lines.[119] Like the inflammatory models, these were used to study disease progression and targeted therapeutic interventions. Mohapatra et al[117] used the cyclin-dependent kinase inhibitor roscovitine to inhibit growth of the A375 cells within the dermal layer of the 3-D matrix. Using combination therapies, Vörsmann et al[118] showed significant advantages in using their 3-D melanoma model to deliver in vivo-like responses compared to a standard 2-D monolayer culture.

A novel chimeric model consisting of a human artificial 3-D skin construct grafted onto the back of SCID mice has also been reported.[120] The bioengineered skin, containing human keratinocytes and fibroblasts isolated from skin biopsies of healthy donors or scleroderma patients, was generated ex vivo and then grafted onto the back of SCID mice. Results implicated the involvement of a PDGF receptor-mediated pathway in the disease and confirmed the suitability for testing in vivo the disease progression-screening antifibrotic drugs.

Summary and conclusion

Despite ethical concerns, the use of animals or isolated animal skin models to assess percutaneous absorption of molecules is frequently reported. These models are generally more widely available than human skin, and prove important in basic research to improve our understanding of the processes, pathways, and driving forces of various agents across the skin barrier. However, because of a large number of animal skin models described in the literature, it may be difficult to compare the results obtained across various species, in addition to the variations in experimental methodology used with a specific skin model, such as type of diffusion cells, body site, skin temperature, receiver

media, application dose, and diffusion area. Therefore, it is important to emphasize that in vitro and animal models provide important tools for screening a series of drug formulations, evaluation of skin permeation-enhancing properties and mechanism of action of the carrier systems, and estimation of rank of skin transport for a series of drug molecules. Also, the majority of the work on synthetic membranes for transdermal and topical delivery studies has been focused on the use of polymeric materials, usually silicone based. Such membranes are ideal for replacing ex vivo skin, as they can be prepared with a defined thickness, are easy to handle and store, are comparatively cheap, inert, and provide reproducible results. Despite all of these advantages, they cannot completely replace human or animal skin for prediction of skin absorption in vivo. These membranes generally lack the type of barrier normally provided by the SC in ex vivo or in vivo skin, and this may lead to some false-positive results in toxicity studies and permeation studies. Therefore, we recommend that where possible, human skin should be used in skin-permeation studies.

A wide range of skin models for testing skin absorption for cutaneous and transdermal delivery has been developed. There is an increasing need, largely driven by regulatory authorities and industry, to ensure that the models and testing protocols are standardized and reproducible, and are validated to show that they accurately reflect the in vivo situation.

Disclosure

The authors report no conflicts of interest in this work.

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EXHIBIT 3

BlueWillow Biologics® Launches NanoBio® Protect Nasal Antiseptic Solution

Provides Next-Level Protection to the Nose, A Main Gateway for Infection-Causing Germs

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NanoBio Protect Antiseptic Spray

Once applied to the skin in and around the nose, the key entry point for most respiratory infections, NanoBio Protect effectively provides next-level protection for up to 8 hours. NanoBio is easy to apply with a cotton swab, pleasant to use, and the moisturizing, alcohol- and fragrance-free formula is safe for the entire family. Each 0.61fl oz (18mL) bottle provides at least 40 treatments and is currently available for purchase on Amazon.com. NanoBio Protect will also be launching in over 4,400 CVS Pharmacy stores across the U.S. beginning in November 2020.

NanoBio Protect's broad-spectrum effectiveness stems from the company's proprietary nanotechnology. The patented, natural oil nanodroplets optimize the antiseptic's (0.13% Benzalkonium chloride [BZK]) antimicrobial activity and its ability to kill germs on the skin. NanoBio® Protect works because it is scientifically formulated to be the right size and charge to kill infection-causing germs in and around the nose where they are most likely to enter the body. Uniquely, the positively charged nanodroplets remain active on the skin for up to 8 hours, attracting the negatively charged germs and killing them on contact. This phenomenon, fondly referred to by the BlueWillow scientists as the "*Dance of the Nanodroplets*," is why the product incomparably provides safe and long-lasting protection from infection.

To help NanoBio Protect fulfill its mission to help people breathe easier and live healthier lives, BlueWillow has partnered with Good360, the global leader in product philanthropy and purposeful giving, whose mission is to transform lives of those impacted by disasters or other challenging life circumstances, including the COVID-19 pandemic.

Beginning October 8, 2020, for every bottle of NanoBio Protect purchased, one bottle will be donated to Good360. In turn, Good360 will distribute NanoBio Protect to families in need and frontline workers serving economically disadvantaged and high-risk communities.

Dr. Chad Costley, physician and CEO of BlueWillow Biologics stated, "We are thrilled to launch the first-of-its-kind nasal antiseptic, NanoBio Protect, a truly revolutionary product." "It couldn't come at a more crucial time as the need to protect ourselves and our families from infection becomes even more vital," he added.

Studies* show the average person touches their face an average of 23 times per hour, providing a lot of opportunity for germs to get in the nose. Applying NanoBio Protect every day – along with frequent handwashing, wearing face coverings, and practicing distancing – is an *essential* part of a daily regimen to protect, prevent and stop the spread of contagious respiratory infections. NanoBio can play an essential role in staying healthy as we resume normal activities such as going back to school, work, travel, dining, shopping, gyms, social events and more where we are likely to pick up germs.

About BlueWillow Biologics

BlueWillow Biologics is a clinical-stage, privately-held biopharmaceutical company focused on developing and bringing to market topical antiseptics, anti-infective products, and intranasal vaccines using its proprietary nanotechnology platform. The innovative, patented oil-in-water nanodroplets are employed in **NanoBio Protect**, the first over-the-counter, alcohol-free nasal antiseptic proven 99.99% effective to kill germs on the skin in and around the nose that cause respiratory infections. Visit www.nextlevelprotection.net for more information.

BlueWillow is also at the forefront of developing intranasal vaccines using its exclusive NanoVax® platform that elicit both systemic and mucosal immunity for several respiratory and sexually transmitted infections, including Sars-CoV-2 (Covid-19), RSV, HSV2, pandemic influenza, and anthrax, as well as intranasal immunotherapy for peanut allergy and other allergic conditions.

*[Source: Kwok et al. Am J Infect Control. 2015 Feb;43(2):112-4. doi: 10.1016/j.ajic.2014.10.015. Face Touching: A Frequent Habit That Has Implications for Hand Hygiene. https://www.ajicjournal.org/article/S0196-6553(14)01281-4/fulltext]

SOURCE BlueWillow Biologics

Related Links

www.bluewillow.com

EXHIBIT 4

WARNING LETTER

Blue Willow Biologics

MARCS-CMS 613948 — AUGUST 03, 2021

Product:

Drugs

Recipient:

Chad Costley, MD/MBA

President and Chief Executive Officer

Blue Willow Biologics

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Ann Arbor, MI 48105
United States

nanobio@bluewillow.com (mailto:nanobio@bluewillow.com)

Issuing Office:

Center for Drug Evaluation and Research

United States

WARNING LETTER

Date: August 3, 2021

RE: Unapproved New Drug Products Related to Coronavirus Disease 2019 (COVID-19)

This is to advise you that the United States Food and Drug Administration (FDA) had reviewed your websites at the Internet addresses https://nanobioprotect.com and https://bluewillow.com on March 24, 2021. The FDA observed that your websites offered a non-alcohol-based consumer antiseptic nasal product[1] for sale in the United States and that this product was intended to mitigate, prevent, treat, diagnose, or cure COVID-19[2] and other conditions in people. FDA also observed that your product is for sale in the United States on other websites, including at https://www.cvs.com/shop/nanobio-protect-nasal-antiseptic-prodid-160333. Based on our review, this product is an unapproved new drug introduced or delivered for introduction into interstate commerce in violation of section 505(a) of the Federal Food, Drug, and Cosmetic Act (FD&C Act), 21 U.S.C. § 355(a). Furthermore, this product is a misbranded drug under sections 502(a) and (ee) of the FD&C Act, 21 U.S.C. 352(a) and (ee). Introduction or delivery for introduction of such a product into interstate commerce violated sections 301(a) and (d) of the FD&C Act, 21 U.S.C. 331(a) and (d). These violations are described in more detail below.

There is currently a global outbreak of respiratory disease caused by a novel coronavirus that has been named "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2). The disease caused by the virus has been named "Coronavirus Disease 2019" (COVID-19). On January 31, 2020, the Department of Health and Human Services (HHS) issued a declaration of a public health emergency related to COVID-19 and mobilized the Operating Divisions of HHS.[3] In addition, on March 13, 2020, there was a Presidential declaration of a national emergency in response to COVID-19.[4] Therefore, FDA is taking urgent measures to protect consumers from certain products that, without approval or authorization by FDA, claim to mitigate, prevent, treat, diagnose, or cure COVID-19 in people. As described below, you sold an unapproved and unauthorized product that was intended to mitigate, prevent, treat, diagnose, or cure COVID-19 and other conditions in people.

Some examples of the claims on your product label that establish the intended use of your product (as defined in 21 CFR 201.128) and examples of the claims on your website that established the intended use of your product and misleadingly represented it as safe and/or effective for the treatment or prevention of COVID-19 and other serious infections include, but may not be limited to, the following:

NanoBioProtect.com and Bluewillow.com websites and NanoBio Protect Nasal Antiseptic label:

- NanoBio® Protect Over-The-Counter Nasal Antiseptic Kills COVID-19 Virus in Lab Tests." [from your website https://bluewillow.com/%ef%bb%bf%ef%bb%bfnanobio-protect-over-the-counter-nasal-antiseptic-kills-covid-19-virus-in-lab-tests/]
- "Recent studies conducted by Public Health England also demonstrate NanoBio® Protect's ability to kill COVID-19 virus in laboratory test." [from your website https://bluewillow.com/nanobio-protect/]
- "NanoBio Protect . . . Protects for up to 8 hours . . . Kills 99.99% of germs on contact . . . Reduces the risk of respiratory infection …." [from your website https://nanobioprotect.com/]
- "KILLS GERMS THAT ENTER THE NOSE . . . PROTECTS FOR 8 HOURS . . .NEXT-LEVEL PROTECTION AGAINST INFECTION" [from your product label]
- "Get protection from infection that lasts all day." [from your website https://nanobioprotect.com/]
- "NanoBio Protect offers next-level protection to help keep you and your family healthy. It is the only alcohol-free nasal antiseptic proven to kill 99.99% of infection-causing germs when applied to the skin in and around the nose. . . NanoBio Protect uses patented, natural-oil nanodroplets that deliver the antiseptic directly to the surface of germs, killing them on contact. Uniquely, the nanodroplets remain active on the skin after application for up to 8 hours (in lab testing), providing lasting protection against the germs that cause infection." [from your website https://nanobioprotect.com/frequently-asked-questions/]
- "NanoBio Protect adds BZK antiseptic to the surface of nanodroplets. This technology offers four distinct advantages over conventional BZK antiseptics:

The Nanodroplets optimize the ability of the antiseptic to kill germs. The droplets sit on skin after application, enabling protection for up to 8 hours (in lab testing) . . ." [from your website https://nanobioprotect.com/frequently-asked-questions/]

- "Is NanoBio Protect US FDA regulated? Yes. NanoBio® Protect nasal antiseptic adheres to the OTC monograph system for topical skin antiseptics. In testing by independent FDA registered laboratories, NanoBio® Protect was shown to kill 99.99% of a broad spectrum of pathogenic organisms. To learn

more, visit https://bluewillow.com/nanobio-protect/." [from your website https://nanobioprotect.com/frequently-asked-questions/]

We note that, as of the date of this letter, some of the claims cited above appear to have been removed. Due to the serious public health concerns related to the marketing and sale of unapproved drugs for the mitigation, prevention, treatment, diagnosis, or cure of COVID-19, it is essential that these violations do not recur.

Based on the above claims, your topical antiseptic product is a drug as defined by section 201(g)(1)(B) of the FD&C Act, 21 U.S.C. § 321(g)(1)(B), because it is intended for the diagnosis, cure, mitigation, treatment, or prevention of disease, and/or under section 201(g)(1)(C) of the FD&C Act, 21 U.S.C. § 321(g)(1)(C), because it is intended to affect the structure or any function of the body. Specifically, your product is intended for use as a consumer topical antiseptic.

This consumer topical antiseptic is a "new drug" within the meaning of section 201(p) of the FD&C Act, 21 U.S.C. 321(p), because it is not generally recognized as safe and effective (GRASE) for use under the conditions prescribed, recommended, or suggested in its labeling. New drugs may not be introduced or delivered for introduction into interstate commerce without prior approval from FDA, as described in section 505(a) of the FD&C Act, 21 U.S.C. 355(a), unless they are lawfully marketed under section 505G of the FD&C Act (which is not the case for this product, as further described below) or under other exceptions not applicable here. No FDA-approved application pursuant to section 505 of the FD&C Act, 21 U.S.C. 355, is in effect for this drug product, nor are we aware of any adequate and well-controlled clinical studies in the published literature that support a determination that your NanoBio Protect Nasal Antiseptic Solution product is GRASE for use under the conditions suggested, recommended, or prescribed in its labeling. Accordingly, this product is an unapproved new drug marketed in violation of sections 505(a) and 301(d) of the FD&C Act, 21 U.S.C 355(a) and 331(d).

We note that over-the-counter (OTC) topical antiseptic products had been the subject of rulemaking under the Agency's OTC Drug Review. In particular, such products were addressed in a tentative final monograph (TFM) entitled "Topical Antimicrobial Drug Products for Over-the-Counter Human Use; Tentative Final Monograph for Health-Care Antiseptic Drug Products," Proposed Rule, 59 FR 31402 (June 17, 1994) (1994 TFM), as further amended by "Safety and Effectiveness of Consumer Antiseptics; Topical Antimicrobial Drug Products for Over-the-Counter Human Use; Proposed Amendment of the Tentative Final Monograph; Reopening of Administrative Record," Proposed Rule, 81 FR 42912 (June 30, 2016)(Consumer Antiseptic Rubs Proposed Rule). Over the course of these rulemakings, three active ingredients (benzalkonium chloride, ethyl alcohol (ethanol), and isopropyl alcohol) were classified as Category III for use in consumer antiseptic rub products, meaning that additional safety and effectiveness data are needed to support a determination that a drug product containing one of these active ingredients would be GRASE for use as a consumer antiseptic rub.

Section 505G of the FD&C Act addresses nonprescription drugs marketed without an approved application. Under section 505G(a)(3) of the FD&C Act, drugs that were classified as Category III for safety or effectiveness in a TFM that is the most recently applicable proposal or determination for such drug issued under 21 CFR Part 330 – and that were not classified as Category II for safety or effectiveness – are not required to have an approved application under section 505 in order to be marketed, as long as they are in conformity with the relevant conditions of use outlined in the applicable TFM, including the active ingredient, and comply with all other applicable requirements.

However, your consumer topical antiseptic product does not conform to the 1994 TFM, as further amended by the 2016 Consumer Antiseptic Rubs Proposed Rule, nor any other TFM, proposed rule, or final rule, and does not meet the conditions under section 505G(a)(3) of the FD&C Act for marketing without an approved application under section 505.

Specifically, your labeling claims suggesting and depicting that your topical antiseptic product is effective in preventing infection or disease go beyond merely describing the general intended use of consumer antiseptics as set forth in the 1994 TFM.5 Your claims on the product label, suggesting your consumer antiseptic provides all day efficacy against infection, and your claims on your websites, which suggested all day protection against serious-disease related pathogens including the novel coronavirus that causes COVID-19, do not conform to the 1994 TFM or any of the amendments to the TFM discussed above. Time-specific extended efficacy claims, especially when related to serious-disease related pathogens, endanger the public health by creating a false sense of security for the general public that may result in infrequent hand washing or the substitution of these products for protective gloves and clothing, which are the principal methods for protecting against the spread of diseases caused by pathogenic microorganisms. As a result, this product may have given users the false impression that they need not rigorously adhere to interventions such as social distancing and exercising good hygienic practices that have been demonstrated to curb the spread of COVID-19. Users who did not follow these interventions were at increased risk for contracting COVID-19 and for spreading disease if they had been exposed to the virus, thereby prolonging the pandemic, and increasing its associated morbidity and mortality. In addition, according to the product labeling, NanoBio Protect Nasal Antiseptic Solution is intended to be applied inside and around the nostrils. Consumer antiseptic products intended for administration inside and around the nostrils are not permitted under the 1994 TFM, as further amended by the 2016 Consumer Antiseptic Rubs Proposed Rule[6].

We are unaware of any adequate and well-controlled clinical studies in the published literature that support a determination that your consumer topical antiseptic product is GRASE for the above-described intended uses, including the time-specific extended efficacy claims. Accordingly, your consumer topical antiseptic product is a new drug under section 201(p) of the FD&C Act. In addition, there are no FDA-approved applications in effect for your consumer topical antiseptic product and, accordingly, it is an unapproved new drug sold in violation of sections 505(a) and 301(d) of the FD&C Act, 21 U.S.C §§ 355(a) and 331(d). We note that your consumer topical antiseptic product also does not conform to any temporary policy FDA has implemented for alcohol-based hand sanitizer products during the public health emergency.[7]

This consumer topical antiseptic product was also misbranded under section 502(a) of the FD&C Act, 21 U.S.C 352(a), because the labeling was false or misleading. Specifically, labeling for this product claimed that the product “adheres to the OTC monograph system for topical skin antiseptics.” As noted above, this product does not meet the conditions under section 505G(a)(3) of the FD&C Act for marketing without an approved application under section 505.

This consumer topical antiseptic products is also misbranded under section 502(ee) of the FD&C Act, 21 U.S.C. 352(ee), because it is a nonprescription drug subject to section 505G of the FD&C Act, 21 U.S.C. 355h, but does not comply with the requirements for marketing under that section and is not the subject of an application approved under section 505 of the FD&C Act, 21 U.S.C. 355.

The introduction or delivery for introduction of a misbranded drug into interstate commerce is prohibited under section 301(a) of the FD&C Act, 21 U.S.C. 331(a).

You should take immediate action to address the violations cited in this letter. This letter is not meant to be an all-inclusive list of violations that exist in connection with your products or operations. It is your responsibility to ensure that the products you sell are in compliance with the FD&C Act and FDA's implementing regulations. We advise you to review your websites, product labels, and other labeling and promotional materials to ensure that you are not misleadingly representing your products as safe and effective for a COVID-19-related use for which they have not been approved by FDA and that you do not make claims that misbrand the products in violation of the FD&C Act. **Within 48 hours, please send an email to COVID-19-Task-Force-CDER@fda.hhs.gov** describing the specific steps you have taken to prevent the recurrence of future violations. Failure to adequately correct any violations may result in legal action, including, without limitation, seizure, and injunction.

FDA is advising consumers not to purchase or use certain products that have not been approved, cleared, or authorized by FDA and that are being misleadingly represented as safe and/or effective for the treatment or prevention of COVID-19. Your firm will be added to a published list on FDA's website of firms and websites that have received warning letters from FDA concerning the sale or distribution of COVID-19 related products in violation of the FD&C Act, with a notation that, as of the date of this letter, your firm's listed products are no longer sold for the prevention, treatment, mitigation, diagnosis or cure of COVID-19. This list can be found at http://www.fda.gov/consumers/health-fraud-scams/fraudulent-coronavirus-disease-covid-19-products (http://www.fda.gov/consumers/health-fraud-scams/fraudulent-coronavirus-disease-covid-19-products).Corrective actions taken to address the sale of your unapproved and unauthorized product for the mitigation, prevention, treatment, diagnosis, or cure of COVID-19, and any appropriate corrective actions have to be confirmed by the FDA, for the published list to be updated to indicate that your firm has taken such corrective actions.

This letter notifies you of our concerns and provides you with an opportunity to address them. If you cannot complete corrective action within 48 hours, state the reason for the delay and the time within which you will complete the corrections. If you believe that your products are not in violation of the FD&C Act, include your reasoning and any supporting information for our consideration.

If you are not located in the United States, please note that products that appear to be misbranded or unapproved new drugs are subject to detention and refusal of admission if they are offered for importation into the United States. We may advise the appropriate regulatory officials in the country from which you operate that FDA considers your product(s) referenced above to be unapproved and misbranded products that cannot be legally sold to consumers in the United States.

Please direct any inquiries to FDA at COVID-19-Task-Force-CDER@fda.hhs.gov.

Sincerely,
/S/
Donald D. Ashley
Director
Office of Compliance
Center for Drug Evaluation and Research
Food and Drug Administration

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1 Your non-alcohol-based consumer antiseptic nasal product is NanoBio Protect Nasal Antiseptic Solution.

2 As explained in the next paragraph, there is currently an outbreak of a respiratory disease named "Coronavirus Disease 2019" (COVID-19).

3 Secretary of Health and Human Services, Determination that a Public Health Emergency Exists. (originally issued on Jan. 31, 2020 and subsequently renewed) available at https://www.phe.gov/emergency/news/healthactions/phe/Pages/2019-nCoV.aspx (https://www.phe.gov/emergency/news/healthactions/phe/Pages/2019-nCoV.aspx).

4 Proclamation on Declaring a National Emergency Concerning the Novel Coronavirus Disease (COVID-19) Outbreak. (Mar. 13, 2020), available at https://www.whitehouse.gov/presidential-actions/proclamation-declaring-national-emergency-concerning-novel-coronavirus-disease-covid-19-outbreak/ (https://www.whitehouse.gov/presidential-actions/proclamation-declaring-national-emergency-concerning-novel-coronavirus-disease-covid-19-outbreak/)).

5 The 1994 TFM covers health care antiseptics that are indicated for use to help reduce bacteria that potentially can cause disease and health care and consumer antiseptics that are indicated for use to decrease bacteria on the skin. 59 FR at 31443.

6 The 2016 Consumer Antiseptic Rubs Proposed Rule covered consumer antiseptic products intended for use of without water. Under the 1994 TFM, as amended by the 2016 Consumer Antiseptic Rubs Proposed Rule, only consumer topical antiseptic products intended for use on the hands without water are permitted. Products intended for other areas of the body such as the nose are not permitted.

7 See, e.g., *Temporary Policy for Preparation of Certain Alcohol-Based Hand Sanitizer Products During the Public Health Emergency (COVID-19)*. Because your consumer topical antiseptic product is not consistent with the formulations described in these guidances, it does not fall within any temporary Agency policy not to take action against firms manufacturing hand sanitizer products for violations of section 505 of the FD&C Act.

More Warning Letters (/inspections-compliance-enforcement-and-criminal-investigations/compliance-actions-and-activities/warning-letters)

IN THE UNITED STATES DISTRICT COURT

EASTERN DISTRICT OF MICHIGAN

SOUTHERN DIVISION

TRUTEK CORP.,
Plaintiff,

v.

BlueWillow Biologics, Inc.
ROBIN ROE 1 through 10, gender neutral fictitious names, and ABC CORPORATION 1 through 10 (fictitious names).

Defendants.

CIVIL ACTION No. 2:21-cv-10312-SJM-RSW

CERTIFICATE OF SERVICE

Undersigned hereby states that on September 29, 2022, the attorneys for Plaintiff caused the foregoing document to be served upon all counsel of record, via electronic service.

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